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# THE DISTINCTIVE FEATURES OF THE TUMOURS OF THE SALIVARY GLANDS IN MALAYA.

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THIS paper is principally concerned with the so-called mixed tumour of the salivary glands, the other types of tumours which may occur in the salivary glands being as rare in Malaya as they are elsewhere, and apparently presenting no special differences. There seems to be fairly general agreement to-day that the mixed tumour is not a mixed tumour at all, but merely a special sort of adenoma, but unfortunately there is no such general agreement as to what these tumours should be called, so that " mixed tumour " continues to be used, however apologetically, as the only term likely to be generally understood. The present paper is concerned with the differences in incidence and site of these tumours in Malaya, as revealed by a study of 60 cases which have been seen in the last few years at this Institute. Such racial differences in the incidence of neoplasms should shed some light on the aetiology; in the present case the suggestion is made that the chronic salivary gland enlargement of malignant malnutrition may predispose to the subsequent development of this neoplasm.

*General and racial incidence.*

In a country such as Malaya it is impossible to say what the true incidence of any neoplasm is, because an unknown but almost certainly large number of cases are never seen by a medical practitioner. The only guide that we have in this matter, and it is a very uncertain guide, is that less than 30 per cent of all deaths are certified by a medical practitioner. However, when a certain number of cases of a particular neoplasm are seen in a year we can say that at least that number occurred, and that the true incidence is probably higher. The mixed tumour of the salivary glands seems to be commoner in Malaya than in Britain; eighteen cases were seen in this Institute in 1950, and 15 in 1949, which was in each case about 4·5 per cent of all neoplasms examined. The 60 cases were distributed racially as shown in Table I; the remaining 1 per cent of the population and 2 per cent of hospital in-patients comprise the other races in Malaya, the figures being based on the 1947 Census.

TABLE I.—*Racial Distribution.*

| | Population (%). | Hospital in-patients (%). | No. of cases. | Percentage of cases. | All neoplasms (IMR. figures) (%). |
|---|---|---|---|---|---|
| Chinese | 39 | 43 | 40 | 67 | 57 |
| Malay | 49 | 20 | 13 | 21·5 | 12·25 |
| Indian | 11 | 35 | 7 | 11·5 | 30·75 |
| Total | 99 | 98 | 60 | 100 | 100 |

It will be seen that the incidence among Chinese was somewhat greater than would be expected from the apparent incidence of neoplasms in hospital patients of that race, and considerably higher among Malays, while it appears to be less common among Indians. During the period under review no cases were seen among Europeans, and only one case in a Eurasian; of course these are small communities in Malaya, but Europeans provide about 5 per cent of all neoplasms examined, and one might expect to have seen at least one case among them if the incidence had been as high as among Chinese. As was said above, these figures do not necessarily reflect the true incidence, but at least they show that this tumour is quite common in Chinese and Malays, while it is almost certainly much less common in Indians.

*Sex incidence.*

There appears to be no significant difference in sex incidence. The racial figures are: Chinese, 21 males and 19 females; Malays, 7 males and 6 females; Indians, 7 males. The fact that all 7 cases in Indians were males may well be due to chance, or some unknown factor such as a reluctance on the part of Indian women to seek medical advice for such a tumour.

*Age incidence.*

It is impossible to say at what age these tumours commenced; all that can be given is the age at operation, and the tumours had then been present for very varying lengths of time. About half the cases had noticed the presence of a tumour for 5 years or less, while nearly a third had had it for 10 years or more; only 3 cases gave a history of less than 1 year. The age at operation was distributed as shown in Table II.

TABLE II.—*Age at Time of Operation.*

| | Age in years. | | | | | | |
|---|---|---|---|---|---|---|---|
| | 11–20. | 21–30. | 31–40. | 41–50. | 51–60. | 61–70. | Total. |
| Number of cases | 6 | 12 | 16 | 12 | 11 | 3 | 60 |
| Percentage | 10 | 20 | 27 | 20 | 18 | 5 | 100 |
| Percentage given by Willis (1948) | 5 | 20 | 31 | 16 | 13 | 15 | 100 |

Table II does not show any great departure from the usual findings : there seems to be a greater number than usual in the lowest age group, which may be a reflection of a higher general incidence ; the greater number in the group 41 to 60 years is probably due to a longer interval between onset and operation than in Europeans, and the small number over 60 years is almost certainly due to the smaller number of people in this age group in Malaya.

*Site incidence.*

Table III shows the site incidence of these 60 cases.

TABLE III.—*Site Incidence.*

| | Parotid. | Sub-maxillary. | Palate. | Other sites. | Total. |
|---|---|---|---|---|---|
| Chinese | 23 | 14 | 1 | 2 | 40 |
| Malays | 9 | 3 | 1 | 0 | 13 |
| Indians | 6 | 1 | 0 | 0 | 7 |
| Total | 38 | 18 | 2 | 2 | 60 |
| Percentage in each site | 63 | 30 | 3·5 | 3·5 | 100 |
| Percentage recorded by other authors (Willis, 1948) | 83 | 8 | 4 | 5 | 100 |

It will be seen at once that in Malaya a much larger proportion of these tumours arises in the submaxillary salivary gland than is the case elsewhere. The published series of cases from Australia, Germany, Britain and the United States all show an incidence of these tumours in the submaxillary salivary glands of rather under 10 per cent, and this 10 per cent incidence in the submaxillary gland has been accepted as the natural distribution of tumour occurrence by both Harvey, Dawson and Innes (1940) and Willis (1948). But in Malaya the incidence in the submaxillary gland is about 30 per cent, and there must be some reason for this striking difference.

The figures are rather inadequate for a proper consideration of the racial differences in site incidence ; the increased incidence in the submaxillary salivary gland is undoubted in the Chinese ; the number of cases among the Malays is very small, but at least the figures are consistent with the view that there is a similar increased incidence among them. The number of cases in Indians is too small for any conclusion to be drawn, but the figures as they stand do not show any increased incidence of submaxillary tumours.

There appears to be no difference in the incidence in the palate and other minor sites, possibly because the factor responsible for the increased incidence in the submaxillary gland does not affect these minor salivary glands, or, more probably, because the figures are too small to show an increase in these less common sites. A paper recently published by Monro (1950) shows a similar site incidence in Singapore. The population of Singapore is predominantly Chinese so that the figures of racial incidence are not of great value, but both the high incidence of the mixed salivary tumour and the high proportion of cases in which it arises

in the submaxillary salivary gland are well shown. The numbers are small, but there seems also to be some increase in the incidence in the minor sites such as the palate.

*Malignancy.*

Many standard text-books of pathology still describe the mixed salivary tumour as benign; certainly metastasis is rare, but recurrence is common, and these recurrences are not uncommonly locally invasive, as indeed the original growth may be also. Malignant behaviour is too frequent for them to be described as benign and not sufficiently frequent for them to be called malignant; each case must be classified separately on its merits.

The recurrence rate is not known in Malaya. Of these 60 cases only 3 were recurrences, but this is almost certainly not the true rate. It seems probable that a recurrence would be considered a failure of the methods of Western medicine, and it would be considered very foolish to try again a method of treatment which had already proved a failure.

Six of these 60 cases showed evidence of malignancy, 4 were locally invasive and in 2 there was metastasis to the cervical glands. A 10 per cent malignancy rate seems high, certainly higher than is found among Europeans, but the numbers are small and the true rate may not be as high as this. The malignant cases are recorded briefly as showing some points of interest.

*Case* 1.—A Malay male aged 66 years. History of a tumour of the parotid since childhood; in the last 2 years it suddenly began to grow rapidly. Tumour was found to be mainly encapsulated but was invading the structures on its deep surface.

*Case* 2.—A male Chinese aged 45 years. Ten years' history of a tumour in the cheek. Tumour was not encapsulated and was invading the surrounding tissues.

*Case* 3.—A male Indian aged 35 years. Short history of parotid tumour. No capsule, facial paralysis, tumour adherent to bone. Invading muscle.

*Case* 4.—A male Indian aged 60 years. Tumour of parotid of unknown duration but now growing rapidly. Found to be invading muscle.

*Case* 5.—A female Chinese aged 65 years. Tumour of parotid excised 3 years ago, but has now recurred and is growing rapidly. Metastatic deposits present in cervical glands.

*Case* 6.—A Malay girl aged 18 years. Large parotid tumour mainly encapsulated but adherent to mastoid process. Metastatic deposits in 3 cervical glands.

It will be seen that only one of the cases was a recurrence. There are frequent references to the possibility of recurrences showing evidence of malignancy as if only recurrences could be malignant; this of course is not so and there is no reason why it should be. In 2 of the cases there was a long history, in 1 of them very long, the tumour after many years of quiescence suddenly beginning to grow rapidly. In the other 2 cases the history was short and the tumour had grown rapidly from the first, so that in these neoplasms, as in many others, malignancy may develop after years of apparently benign growth, or they may be frankly malignant from their inception.

Of the 2 cases in which metastasis occurred, 1 was a recurrence with increased rate of growth in an elderly woman, the other was a young girl with a large tumour

which was mainly encapsulated. In the former there had been an increased rate of growth, in the latter it had always been rapid.

There seems to be a tendency for the age of these malignant cases to be higher than the average for the benign forms, half of the cases being 60 years old or more. On the other hand one malignant case was a girl of 18 years ; metastasis in these tumours is always rare, but it has been recorded before in young people.

There appears to be no special racial incidence of these cases, and in all but one case the tumour arose in the parotid gland.

*Other forms of salivary tumour.*

These appear to be as uncommon in Malaya as elsewhere, only 2 cases being seen in the period under review. There were two cases of adenolymphoma, both in male Malays. There was also one case of anaplastic carcinoma of the parotid gland in which there was no evidence of a pre-existing " mixed " salivary tumour, so it was not included in the series.

## DISCUSSION.

It seems certain that there is a higher incidence of the so-called " mixed " salivary tumour in Malaya than is found in European races. There is no evidence that this increased incidence is found in Indians ; it appears to be confined to Chinese and Malays, and perhaps it is greatest in Malays. It is stated by Gillman, Gilbert and Gillman (1947) that there is also a higher incidence of this tumour in negroes, although other neoplasms of the salivary gland are rare. It is thought that this is associated with the enlargement of the salivary glands which is part of the syndrome known as kwashiorkor or malignant malnutrition. This condition is characterized by a fatty liver, lesions in the pancreas and kidneys, and enlargement of the salivary glands. At first it was considered to be a disease of infants, but Trowell (1948*a*) extended the term to include a similar condition in adults, and emphasized that malignant malnutrition in childhood leads to certain permanent and incurable defects. He described the lesions as " chronic irritative lesions and death of parenchymal cells as well as a tendency towards carcinoma in the liver and pancreas " (Trowell, 1948*b*). This condition has also been described in Brazil, Budapest, Curaçao and among prisoners of war in Malaya and Siam, but no reference is made to any increased incidence of mixed salivary tumours.

Malignant malnutrition is found in infants and children in the poorer areas of Malaya. The Senior Nutrition Officer at this Institute in a personal communication gives the following figures for enlargement of the parotid glands among school children in the poorer areas : Malays 20 per cent., Chinese 10 per cent, Indians 5 per cent. The submaxillary glands are certainly frequently and probably always also enlarged, but the parotid gland is taken as a standard because it is more easily observed. This enlargement of the parotid glands occurs in childhood, but becomes chronic and persists into adult life or even becomes permanent. Unfortunately nothing is known of the histological basis for this chronic enlargement of the salivary glands in Malaya. Gillman, Gilbert and Gillman (1947) in Africa state that the enlargement of the parotid gland is due to an increase in size of the individual gland cells, with perhaps an interstitial accumulation of fat. They state that the changes in the submaxillary and sublingual glands resembled those in the liver, and included

atrophy, fibrosis and cytosiderosis, although fatty change was less common. They further state that parotid changes were found almost exclusively in males. In Malaya at least parotid enlargement is equally common in males and females.

Another important difference in the mixed salivary tumour in Malaya is the much higher incidence of these tumours in the submaxillary gland than is found elsewhere; in Malaya about 30 per cent occur in the submaxillary salivary gland, while all published series from other countries agree in showing only about 10 per cent. This fact becomes more suggestive when we consider the relative sizes of the parotid and the submaxillary salivary glands: 'Gray's Anatomy' gives their weights as 25 g. and 8 g. respectively, so that the submaxillary gland is just under a third of the size of the parotid. When we compare this with the site incidence of mixed salivary tumours in Malaya it will be seen that there appears to be an equal liability for this neoplasm to arise in either the parotid or the submaxillary salivary gland, the different numbers observed appearing merely proportional to the different amounts of tissue in the two glands.

This observation suggests that there must be a different factor operative in European races to account for the special liability of the parotid gland to undergo neoplastic change. Willis (1948) has suggested that the special liability of the parotid gland to this form of neoplasm is due to the greater number of serous cells in it; this may well be true, but if so, the aetiology of this tumour in Malays and Chinese must be quite different. Here both parotid and submaxillary salivary tissue appears equally liable to undergo neoplastic change, and the frequency of such change is considerably greater than in European races. This seems to indicate the presence of another cause for this neoplasm in Malaya; it may be that the cause of this tumour in European races is not present at all in Malaya, and that all cases are due to a Malayan factor which affects all salivary tissue equally, but this seems very improbable. It is more likely that the European factor is also present but is of only minor importance, the majority of cases being due to this Malayan factor, in which case it must show some predilection for the submaxillary salivary gland. This might be explained if the different histological changes found in the submaxillary salivary gland in malignant malnutrition by Gillman, Gilbert and Gillman (1947) in Africa were also present here. It is, of course, pure speculation to suggest that the special factor operative in Malaya is malignant malnutrition, but there are some suggestive facts to support it. In Africa and in Malaya it is known that where malignant malnutrition is common so is the mixed salivary tumour. In Africa it is claimed that the changes in the liver and pancreas resulting from malignant malnutrition predispose to the subsequent development of carcinoma; if this is true it seems probable that there would be a similar predisposition to neoplastic change in the salivary glands. Finally the racial incidence of malignant malnutrition in Malaya bears some resemblance to the racial incidence of mixed salivary tumour.

There appears to be no increase in the incidence of the salivary adenolymphoma; Gillman, Gilbert and Gillman (1947) record a similar finding from South Africa; so whatever the cause of the increased incidence of the mixed tumour, it does not operate for the adenolymphoma. If Nicholson's (1923) view that these tumours arise in heterotopic salivary tissue in lymph glands is correct, it might explain why they are not affected. Supposing again that the salivary gland changes of malignant malnutrition do predispose to the mixed

salivary tumour, it might well not affect such heterotopic tissue, the changes being confined to the functioning glands.

SUMMARY.

A series of 60 cases of the so-called mixed salivary gland tumour is recorded. It is thought that this shows an unusually high incidence of this neoplasm in Malaya, and that there are definite racial differences in incidence, the majority of cases occurring in Chinese and Malays.

Approximately 30 per cent of these tumours occurred in the submaxillary salivary glands, whereas series from other countries record only 10 per cent.

These findings are discussed, and the possibility of their association with the chronic enlargement of the salivary glands resulting from malignant malnutrition is considered.

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# CARCINOMA AND ASBESTOSIS OF THE LUNG : REPORT OF A CASE.

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CURETON (1948) refers to the differences of opinion on the association of asbestosis and carcinoma of the lung. He mentions the statistical difficulties due to the small number of asbestos workers and the paucity of published records of such cases. The following case is therefore recorded :

*Case report.*—D. J—, male, aged 39, had been a ship's steward for five years ; for the previous fifteen years he had kept a green-grocery business ; twenty years ago he had worked for twelve months in an asbestos factory in the East End of London.

In February 1948, he developed a pain in the right side of the chest and dyspnoea. He was in hospital in Cape Town, and a pleural effusion diagnosed. He returned to this country and was admitted to this hospital for investigation. Blood-stained fluid containing a few polymorphonuclear cells and lymphocytes was repeatedly aspirated from the right pleural cavity. Both sputum and pleural fluid were repeatedly examined for acid-fast bacilli with negative results. Material aspirated from the bronchus was found, while being examined for malignant cells, to contain typical asbestosis bodies. It was then that inquiry into his previous occupations was made, and the history of work in an asbestos factory obtained. Surgical exploration of the pleural cavity was performed, but the patient died on September 29, 1948.

*Post-mortem examination.*—A large mass of growth was found surrounding and compressing the right lung (Fig. 1), and displacing the heart and mediastinum to the left. A layer of growth 1 to 1½ in. thick covered the lung and the diaphragm, so that the pleural cavity had become quite small. There were large nodular deposits of growth in the glands in the upper mediastinum, around the main vessels, and between the aorta and the oesophagus. The actual site of origin of the growth could not be determined. The left lung was congested and partly collapsed. The abdominal organs showed congestive changes only. No distant metastases were present.

Microscopically the growth is seen to be composed of cuboidal and polygonal cells arranged in glandular cystic spaces, with intracystic papillary formations (Fig. 2). Large numbers of asbestosis bodies can be seen in the lungs.

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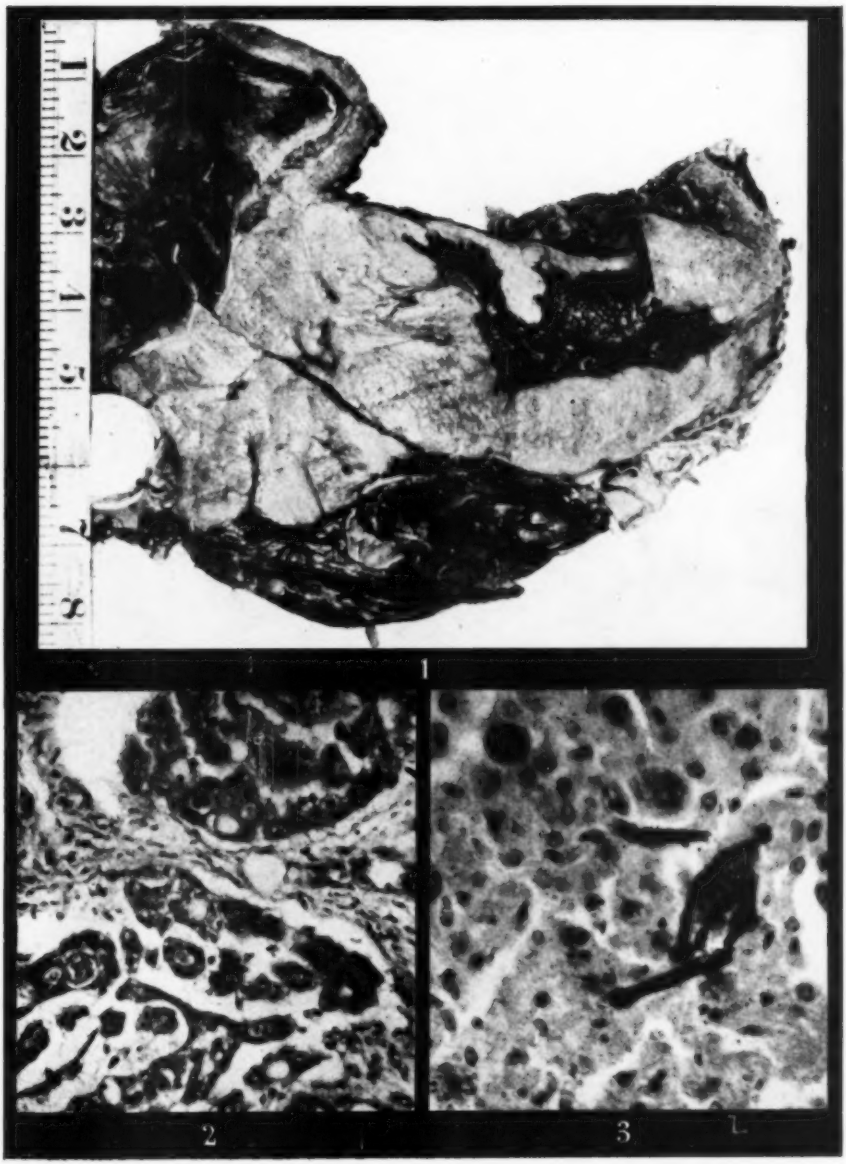
EXPLANATION OF PLATES.

FIG. 1. Growth surrounding the right lung. Scale in inches.

FIG. 2. Growth of the right lung, showing cystic and papillary structure of cuboidal cells.

FIG. 3. Lower lobe of left lung, showing a cluster of asbestosis bodies surrounded by macrophages.



Owen.

Many occur in clusters surrounded by macrophages (Fig. 3), but some are lying free in alveoli.

*Comment.*—The interesting features of this case are the shortness of the exposure to asbestos dust, the long length of time which elapsed before the growth developed, and the unusual histological pattern of the growth. Professor M. J. Stewart (personal communication) states that he is satisfied that there is a statistical relationship between silicosis and asbestosis and cancer, and particularly with the squamous type. Cureton (1948) reviews the published cases up to 1948, and adds one more. This one was of the squamous type, and also had a long latent period, of fifteen years, but the period of exposure, being seven years, was much longer than in the case here reported.

I am indebted to Mr. N. F. Adeney, F.R.C.S. for the clinical notes, and to Professor M. J. Stewart for his opinion.

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# AN EVALUATION OF SOME BIOCHEMICAL TESTS FOR CANCER.

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THE eradication of malignant growths by surgery depends largely upon the early diagnosis of the neoplasm. The need for reliable tests for malignancy has been emphasized by Homberger (1950), who has reviewed more than 60 biochemical, biological and cytological "cancer tests" which have been advocated in the last two decades. Some of these, such as the estimation of serum acid phosphatase (Gutman, 1942; Herbert, 1946) and the cytological examination of fluids (Papanicolaou and Traut, 1943; Shen and Homberger, 1950), are of great value; others, such as the determination of erythrocyte sedimentation rate (E.S.R.), are not specific for neoplasia.

The growth of malignant tumours involves an abnormal synthesis of tissue which may be reflected in the serum proteins. Recently serious attention has been given to the qualitative and quantitative changes occurring in serum proteins in neoplasia. Huggins and his colleagues (Huggins, Miller and Jensen, 1949; Huggins, Jensen, Player and Hospelhorn, 1949; Huggins, Cleveland and Jensen, 1950) have shown that the thermal coagulation of serum proteins, and the inhibition of this coagulation by iodoacetate, is altered in neoplastic disease. Winzler and his colleagues (Winzler, Devor, Mehl and Smyth, 1948; Winzler and Smyth, 1948) have reported that the mucoproteins in serum may be increased in neoplastic disease. Both groups of authors have, however, demonstrated that these tests are not specific for neoplasia. Several reports (Pollak and Leonard, 1950; Bodansky and McInnes, 1950; Homberger, Pfeiffer, Page, Rizzoni and Benotti, 1950; Gilligan, Rothwell and Warren, 1950; Finnegan, Brockland, Muether, Hawk, Inkley and Thoma, 1950) have since been published confirming that some of these tests may not only give positive results in non-malignant cases (i.e., false positive results), but also negative results in malignant cases (i.e., false negative results). Few reports have, however, appeared in which these tests have been used in conjunction with other biochemical tests of known value, such as the estimation of serum acid phosphatase in carcinoma of the prostate and serum alkaline phosphatase in the detection of bone diseases.

This paper records the results of an investigation of the thermal coagulation test, the inhibition of coagulation by iodoacetate and the estimation of serum mucoproteins in 25 normal subjects and 129 patients suffering from malignant neoplasia and other diseases. The serum total protein, albumin and globulin content, the serum phosphatases and E.S.R. have also been estimated for most of the patients and compared with the accepted normal values. A preliminary report of the work was communicated to the Biochemical Society (Broughton, Higgins and O'Brien, 1951).

## METHODS AND MATERIALS.

Patients were chosen, usually before treatment, in whom the diagnosis was proved or likely to be proved. Some with carcinoma, particularly the elderly ones, had complicating diseases, such as chest and urinary infections.

The least concentration of coagulable protein (LCCP) and the iodoacetate index (IAI) were determined by the method of Huggins, Miller and Jensen (1949). In the LCCP test solutions were prepared so that the serum concentration increased in steps of 0·25 ml., and in the IAI test the iodoacetate concentration was increased in steps of 1·5 μM., which were the smallest changes in concentration detectable in our procedure. These solutions were heated in tubes 1 cm. in diameter. In our technique the criterion of coagulation was the formation of a gel sufficiently rigid to slide down the inverted tube after gentle tapping without loss of shape.

Mucoproteins in serum were separated by the method of Winzler, Devor, Mehl and Smyth (1948), and their protein content determined by the biuret method of Weichselbaum (1946).

Serum proteins were determined by the micro-Kjeldahl technique (Howe, 1921). Alkaline phosphatase was determined by the method of King and Armstrong (1934), and acid phosphatase by the method of Gutman and Gutman (1938). The erythrocyte sedimentation rate (E.S.R.) was determined by Westergren's (1921) method. All determinations except the E.S.R. were made on serum, and completed on the day the blood was collected.

The serum flocculation tests used were the thymol turbidity test (Maclagan, 1944*b*), the colloidal gold test (Maclagan, 1944*a*) and the Takata-ara reaction (Ragins, 1934).

## RESULTS.

The following symbols are used in the text and tables :

T = Total protein content of serum (g./100 ml.).
A = Albumin content of serum (g./100 ml.).
G = Globulin content of serum (g./100 ml.).
LCCP = Least concentration of coagulable protein, i.e., the least concentration of serum protein which coagulates on heating (g./100 ml.).
IAI = Iodoacetate index. In this test the end-point is the largest amount of iodoacetate (μM.) which, when added to 0·25 ml. serum, will not inhibit thermal coagulation. Then IAI is this end-point multiplied by 4/T.
P = mg. protein in the mucoprotein present in 100 ml. serum.

B = Biopsy.
L = Laparotomy.
N = Necropsy.
Cl. = Clinical diagnosis.
R = Radiological diagnosis.
M = Malignant cells in pleural fluid.

H = Bronchoscopy.
Ad = Adenocarcinoma.
TC = Transitional-celled carcinoma.
S = Squamous-celled carcinoma.
D = Spheroidal-celled carcinoma.
Y = Leiomyosarcoma.

The numbers in the histology column refer to Broders' (1925) grading. The diagnosis of malignancy was confirmed by laparotomy, necropsy, histological or radiological examination in 80 cases.

TABLE I.—*Normal Ranges for the Least Concentration of Coagulable Protein, the Iodoacetate Index and Serum Mucoprotein Concentration.*

| Test. | No. of analyses. | Range. |
|---|---|---|
| LCCP | 32 | 1·19–1·59 g./100 ml. |
| IAI | 30 | 7·3–13·6 units |
| P | 32 | 40–93 mg./100 ml. |

(For symbols see p. 385.)

*Normal subjects.*

Table I shows the range of values for the thermal coagulation and iodoacetate tests and the estimation of mucoprotein obtained in 32 analyses on a group of 11 females and 14 males, of ages ranging from 20 to 40 years (mean age 26).

The normal range for alkaline phosphatase was taken as 4–14 units/100 ml., total protein 6·3–7·8 g./100 ml., albumin 4·0–5·2 g./100 ml., globulin 1·3–2·9 g./100 ml. (Higgins and O'Brien, unpublished data), acid phosphatase 1–5 units/100 ml. (Gutman and Gutman, 1938), and E.S.R. 1–10 mm./hour (Westergren, 1921).

*Hospital patients.*

Tables II to VIII show the values for the thermal coagulation test, iodoacetate index and mucoprotein concentration, together with the serum proteins, of a group of 82 patients with malignant diseases and 47 patients with non-malignant diseases. The malignant group contained 11 cases of carcinomatosis, 16 neoplasia of bronchus and oesophagus, 15 neoplasia of gastro-intestinal tract, 20 neoplasia of prostate and bladder, 6 neoplasia of biliary tract and 14 miscellaneous malignant diseases. The non-malignant group contained 7 cases of chest infection, 14 benign prostatic hyperplasia, 12 hepatic diseases and 14 miscellaneous diseases.

Table IX records the summary of the results given in Tables II to VIII. It will be seen from this table that none of these tests is completely reliable in the detection of malignancy; all tests gave false positive and false negative results. The LCCP test gave 82 per cent correct positive results in the malignant group, but 62 per cent false positives in the non-malignant group. Similarly the IAI gave 55 per cent correct positive results and 38 per cent false positive results, while the mucoprotein concentration was abnormal in 73 per cent of the malignant cases, but also 48 per cent of the non-malignant cases.

The least concentration of coagulable globulin and the actual amount of iodoacetate added have also been investigated but yielded no additional information.

Youden (1950) has proposed an index for assessing the value of diagnostic tests. These indices were calculated for LCCP, IAI and mucoprotein (P), but differences between them were not significant and they have not therefore been reported.

*Correlation of results.*

None of the three tests, LCCP, IAI and P, detected one histological type of neoplasia better than another, and it was impossible from their results to determine whether the more rapidly growing neoplasms produced greater changes in the serum. Their degree of positivity in cases of widespread carcinomatosis was often no greater than that in cases of single neoplasm.

TABLE II.—*Carcinomatosis.*

(Site of primary growth uncertain.)

| No. | Age. | Sex. | Date. | Metastases. | Proof. | Histology. | LCCP (g./100 ml.). | IAI (units). | P (mg./100 ml.). | Protein. (g./100 ml.) T | A | G | Alk. phos. (units/100 ml.). | E.S.R. (mm./hour). | Notes |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1a | 62 | M. | 19.xii.49 | Stomach, colon, intestines | L | — | 1·68 | 5·3 | 148 | 5·6 | 3·3 | 2·3 | 6 | — | Laparotomy 3.xii.49. |
| 1b | — | — | 11.1.50 | — | — | — | 1·79 | 3·3 | 176 | 5·5 | 3·0 | 2·5 | 12 | — | — |
| 2a | 41 | F. | 18.1.50 | Liver, colon, skin | B | Ad | 2·56 | 2·8 | 172 | 6·4 | 3·4 | 3·0 | 50 | — | Jaundice, oedema. |
| 2b | — | — | 20.ii.50 | — | — | — | 2·17 | 3·9 | 143 | 6·2 | 3·3 | 2·9 | 90 | — | — |
| 3 | 38 | M. | — | Peritoneum, colon, intestines | B | Ad 2–3 | 2·48 | 5·8 | 179 | 6·2 | 3·8 | 2·4 | — | — | Laparotomy 2 days previously. |
| 4 | 48 | M. | — | Stomach, pancreas, spleen | B | Ad 2–3 | 2·85 | 3·0 | 150 | 6·0 | 3·0 | 3·0 | 5 | 44 | — |
| 5 | 41 | F. | — | Colon, peritoneum | B | Ad 2 | 1·99 | 6·8 | 139 | 5·3 | 3·3 | 2·0 | 8 | — | Ascites. |
| 6 | 58 | M. | — | Stomach, liver, spleen | N | — | 1·71 | 4·2 | 160 | 5·7 | 3·3 | 2·4 | 47 | — | Haematemesis. |
| 7 | 71 | M. | — | Liver, intestine, lungs | B, N | Ad | 2·94 | 1·2 | 212 | 5·1 | 2·8 | 2·3 | 8 | — | Laparotomy 3 days previously. |
| 8 | 47 | M. | — | Oesophagus, lungs, intestines | Cl, R | — | 1·72 | 7·8 | 128 | 6·9 | 3·8 | 3·1 | 19 | 88 | — |
| 9 | 70 | M. | — | Bladder, colon, prostate | B | Ad | 2·75 | 3·0 | 246 | 6·1 | 3·2 | 2·9 | 6 | — | — |
| 10 | 56 | M. | — | Colon, liver, intestines | B | Ad | 2·18 | 7·2 | 262 | 6·7 | 4·0 | 2·7 | 17 | — | — |
| 11 | 70 | M. | — | Stomach, pancreas, bones | N | — | 2·00 | 6·3 | 240 | 5·7 | 3·1 | 2·6 | 19 | — | Coronary thrombosis. |

(For symbols see p. 385.)

TABLE III.—*Diseases of Bronchus and Oesophagus.*

| No. | Age. | Sex. | Date. | Metastases. | Proof. | Histology. | LCCP (g./100 ml.). | IAI (units). | P (mg./100 ml.). | Protein. T (g./100 ml.). | Protein. A (g./100 ml.). | Protein. G (g./100 ml.). | Alk. phos. (units/100 ml.). | E.S.R. (mm./hour). | Notes. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (a) *Carcinoma of bronchus.* | | | | | | | | | | | | | | | |
| 12a | 48 | M. | 8.xii.49 | Kidneys, heart | M, N | — | 1·79 | 6·5 | 162 | 5·5 | 3·7 | 1·8 | — | 70 | Hepatic cirrhosis. |
| 12b | — | — | 1.ii.50 | Adrenal | — | — | 2·33 | 6·8 | 193 | 6·2 | 3·6 | 2·6 | 10 | — | — |
| 13a | 38 | M. | 2.1.50 | Liver, spine | Cl, R | — | 2·66 | 3·1 | 258 | 5·9 | 3·4 | 2·5 | 44 | 66 | Pleural effusion. |
| 13b | — | — | 16.1.50 | — | — | — | 3·00 | 2·0 | 231 | 6·0 | 3·3 | 2·7 | 35 | 112 | — |
| 14 | 55 | M. | — | — | B | 83 | 2·28 | 5·1 | 216 | 7·0 | 3·4 | 3·6 | 11 | 124 | — |
| 15 | 63 | M. | — | — | M, R | — | 1·50 | 12·0 | 90 | 6·0 | 3·5 | 2·5 | 9 | 35 | Pleural effusion. |
| 16 | 51 | M. | — | — | H, R | — | 1·93 | 6·2 | 139 | 7·0 | 4·3 | 2·7 | 13 | — | — |
| 17 | 67 | M. | — | — | H, R | — | 1·75 | 10·8 | 125 | 7·8 | 3·8 | 4·0 | 18 | — | — |
| 18 | 60 | M. | — | — | B | 83 | 2·75 | 1·0 | 252 | 6·1 | 2·8 | 3·3 | 21 | 56 | Pleural effusion. |
| 19 | 54 | M. | — | Liver, skin | Cl, R | — | 1·95 | 5·8 | 167 | 5·2 | 3·0 | 2·2 | 48 | — | — |
| 20 | 76 | M. | — | — | Cl, R | — | 2·24 | 4·3 | 224 | 6·9 | 3·3 | 3·6 | 17 | 84 | Bronchitis. |
| 21 | 60 | M. | — | Liver, brain | Cl, R | — | 1·84 | 7·2 | 164 | 6·7 | 3·4 | 3·3 | 59 | 70 | — |
| 22 | 52 | M. | — | — | H, Cl | — | 1·87 | 8·8 | 125 | 7·5 | 3·8 | 3·7 | 12 | 30 | Bronchopneumonia. |
| 23 | 68 | M. | — | Oesophagus | N | — | 2·24 | 5·6 | 174 | 6·4 | 3·2 | 3·2 | 5 | 82 | Bronchopneumonia, hypertension. |
| 24 | 71 | M. | — | — | Cl | — | 2·76 | 2·1 | 241 | 5·8 | 3·7 | 2·1 | 13 | — | Dermatitis. |
| (b) *Carcinoma of oesophagus.* | | | | | | | | | | | | | | | |
| 25 | 73 | M. | — | — | B | 82 | 1·68 | 8·1 | 107 | 6·7 | 4·4 | 2·3 | 4 | — | Cerebral thrombosis. |
| 26 | 71 | F. | — | — | B, N | 82 | 1·65 | 8·2 | 126 | 6·6 | 3·6 | 3·0 | 6 | — | — |
| 27 | 64 | M. | — | — | B, N | 82 | 1·51 | 6·5 | 160 | 5·5 | 3·5 | 2·0 | 8 | — | — |

| No. | Age. | Sex. | Disease. | Proof. | LCCP (g./100 ml.). | IAI (units). | P (mg./100 ml.). | Proteins. T (g./100 ml.). | Proteins. A (g./100 ml.). | Proteins. G (g./100 ml.). | Alk. phos. (units/100 ml.). | E.S.R. (mm./hour). |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (c) *Chest infections.* | | | | | | | | | | | | |
| 106 | 56 | M. | Bronchopneumonia | Cl | 2·22 | 6·2 | 153 | 6·8 | 3·8 | 3·0 | — | — |
| 107 | 57 | M. | Head injury + pneumonia | Cl, N | 1·62 | 8·1 | — | 5·9 | 4·4 | 1·5 | — | — |
| 108 | 69 | M. | Chronic bronchitis | Cl | 2·12 | 6·5 | 135 | 6·5 | 3·3 | 3·2 | — | 3 |
| 109 | 68 | M. | " " | Cl | 1·75 | 6·8 | 122 | 7·0 | 3·2 | 3·8 | 11 | 31 |
| 110 | 19 | M. | Bronchiectasis | Cl | 1·31 | 9·3 | 64 | 5·8 | 4·0 | 1·8 | 9 | 2 |
| 111 | — | F. | " " | Cl | 1·28 | 8·4 | 60 | 6·4 | 4·0 | 2·4 | 10 | — |
| 112 | 54 | M. | Bronchopneumonia + pyelonephritis | Cl, L | 2·22 | 8·1 | 201 | 7·4 | 3·6 | 3·8 | 10 | 77 |

(For symbols see p. 385.)

## TABLE IV.—*Diseases of the Gastro-intestinal Tract.*

| No. | Age. | Sex. | Date. | Metastases. | Proof. | Histology. | LCCP (g./100 ml.). | IAI (units). | P (mg./100 ml.). | Protein. T (g./100 ml.). | Protein. A (g./100 ml.). | Protein. G (g./100 ml.). | Alk. phos. (units/100 ml.). | E.S.R. (mm./hour). | Notes. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (a) *Carcinoma of stomach.* | | | | | | | | | | | | | | | |
| 28a | 51 | F. | 13.xii.49 | — | B | Ad 3–4 | 1·85 | 6·3 | 188 | 5·7 | 3·7 | 2·0 | 3 | 40 | Gastrectomy 13.xii.49. |
| 28b | — | — | 21.xii.49 | — | — | — | 2·29 | 3·9 | 212 | 6·1 | 3·7 | 2·4 | — | — | — |
| 28c | — | — | 11.i.50 | — | — | — | 1·81 | 7·3 | 123 | 6·6 | 4·1 | 2·5 | 10 | — | — |
| 29a | 61 | M. | 13.i.50 | Lymph nodes, colon, pancreas | B | Ad 3 | 1·87 | 6·0 | 146 | 5·0 | 3·1 | 1·9 | 11 | — | Gastrectomy 16.i.50. |
| 29b | — | — | 27.i.50 | — | — | — | 2·79 | 3·4 | 246 | 5·3 | 3·2 | 2·1 | 12 | — | — |
| 30 | 61 | M. | — | *nil* | N | — | 3·26 | 2·9 | 172 | 6·2 | 3·4 | 2·8 | — | 8 | Bronchopneumonia. |
| 31 | 65 | M. | — | — | B | Ad 2–3 | 1·49 | 9·7 | 80 | 6·8 | 4·2 | 2·6 | 14 | 10 | Bronchitis. |
| 32 | 48 | F. | — | Liver, lymph nodes | Cl, R | — | 1·81 | 6·3 | 126 | 5·7 | 3·5 | 2·2 | 38 | 37 | — |
| (b) *Carcinoma of colon.* | | | | | | | | | | | | | | | |
| 33 | 58 | M. | — | Lymph nodes | B | Ad | 1·14 | 10·5 | 74 | 5·7 | 4·0 | 1·7 | 4 | 14 | — |
| 34 | 59 | F. | — | Lymph nodes, intestines | B | Ad | 1·87 | 9·7 | 185 | 6·8 | 4·6 | 2·2 | 18 | — | — |
| 35 | 69 | F. | — | Liver | B | Ad 3 | 1·80 | 7·5 | 117 | 7·2 | 4·2 | 3·0 | 9 | — | Laparotomy 4 days previously. |
| 36 | 39 | F. | — | Ureter | B | Ad 2–3 | 1·83 | 6·9 | 147 | 6·1 | 3·5 | 2·6 | — | 55 | Colostomy 17 days previously. |
| 37 | 66 | F. | — | Peritoneum | L | — | 2·0 | 6·3 | 242 | 5·7 | 3·7 | 2·0 | 4 | — | — |
| (c) *Carcinoma of rectum.* | | | | | | | | | | | | | | | |
| 38 | 62 | F. | — | Lymph nodes, colon | B | Ad 2–3 | 1·89 | 6·7 | 180 | 6·3 | 3·7 | 2·6 | 18 | — | — |
| 39 | 60 | M. | — | — | B | Ad 2–3 | 1·38 | 9·8 | 54 | 5·5 | 4·1 | 1·4 | 16 | 10 | Arteriosclerosis. |
| 40 | 41 | M. | — | — | L | — | 1·62 | 4·3 | 112 | 6·5 | 3·8 | 2·7 | 7 | 38 | — |
| 41a | 59 | F. | 19.xii.49 | Liver, lungs | B, N | Ad 2 | 2·06 | 5·1 | 248 | 5·9 | 3·3 | 2·6 | 47 | — | — |
| 41b | — | — | 11.i.50 | — | — | — | 2·65 | 1·1 | 236 | 5·3 | 3·0 | 2·3 | 58 | — | — |
| 42 | 70 | M. | — | — | B | Ad 2–3 | 1·71 | 7·4 | 87 | 5·7 | 2·4 | 3·3 | — | 10 | — |
| (d) *Non-malignant.* | | | | | | | | | | | | | | | |
| 123 | 69 | M. | — | Duodenal ulcer | N | — | 1·55 | 8·7 | 126 | 6·9 | 3·8 | 3·1 | 7 | — | — |

(For symbols see p. 385.)

TABLE V.—*Diseases of Bladder.*

| No. | Age. | Sex. | Date. | Proof. | Histology. | LCCP (g./100 ml.). | IAI (units). | P (mg./100 ml.). | Protein. T (g./100 ml.) | Protein. A (g./100 ml.) | Protein. G (g./100 ml.) | Alk. phos. (units/100 ml.). | E.S.R. (mm./hour). | Notes. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (a) *Carcinoma.* | | | | | | | | | | | | | | |
| 57a | 55 | M. | 17.i.50 | B | TC2 | 1·57 | 5·1 | 47 | 7·0 | 4·3 | 2·7 | 7 | — | Diabetes, asthma. |
| 57b | — | — | 3.ii.50 | — | — | 1·76 | 7·6 | 132 | 6·3 | 4·0 | 2·3 | 10 | — | Cystectomy 20.i.50. |
| 58 | 65 | M. | — | B | TC | 1·86 | 7·7 | 195 | 6·2 | 3·8 | 2·4 | 7 | 60 | Acute infection. |
| 59 | 69 | M. | — | B | TC1 | 2·16 | 6·7 | 132 | 7·2 | 3·7 | 3·5 | 13 | 32 | Chronic cough. |
| 60 | 71 | M. | — | L | — | 1·80 | 7·5 | 62 | 7·2 | 4·1 | 3·1 | 7 | 7 | — |
| 61 | 66 | M. | — | B, N | S 3–4 | 1·95 | 6·0 | 148 | 6·0 | 3·9 | 2·1 | 12 | — | — |
| 62 | 50 | F. | — | B | S 2–3 | 1·80 | 8·0 | 159 | 6·0 | 3·9 | 2·1 | 8 | — | Rheumatoid arthritis. |
| (b) *Non-malignant.* | | | | | | | | | | | | | | |
| 113a | 45 | M. | 20.i.50 | Cl, B | — | 1·78 | 8·5 | 159 | 7·1 | 4·1 | 3·0 | 9 | — | Pyelonephritis + chronic cystitis. |
| 113b | — | — | 21.ii.50 | — | — | 2·51 | 4·1 | 285 | 5·9 | 3·5 | 2·4 | — | — | — |
| 125 | 62 | M. | — | B | — | 1·45 | 8·3 | 55 | 6·5 | 3·6 | 2·9 | 6 | 2 | Non-malignant transitional celled papilloma of bladder. |
| 126 | 72 | M. | — | Cl | — | 1·95 | 4·6 | 125 | 5·2 | 3·7 | 1·5 | 5 | 24 | Cystitis; emphysema; arteriosclerosis. |

(For symbols see p. 385.)

## TABLE VI.—*Diseases of Prostate.*

| No. | Age. | Date. | Metastases. | Proof. | Histology. | LCCP (g./100 ml.). | IAI (units). | P (mg./100 ml.). | Protein. T (g./100 ml.). | Protein. A (g./100 ml.). | Protein. G (g./100 ml.). | Acid phos. (units/100 ml.). | Alk. phos. (units/100 ml.). | E.S.R. (mm./hour). | Notes. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (a) *Carcinoma of prostate.* | | | | | | | | | | | | | | | |
| 43 | 69 | — | Liver, lymph nodes | B, N | Ad 2 | 2·21 | 4·1 | 140 | 5·9 | 3·0 | 2·9 | 70 | 23 | 27 | After stilboestrol treatment, bronchopneumonia. |
| 44 | 67 | — | Spine, pelvis | B | Ad 1–2 | 2·03 | 6·7 | 116 | 5·4 | 3·5 | 1·9 | 7 | 67 | 35 | After stilboestrol treatment. |
| 45 | 71 | — | — | B | 4 | 1·48 | 7·1 | 87 | 5·9 | 3·7 | 2·2 | 7 | 11 | 10 | " " " |
| 46 | 63 | — | — | B | Ad | 2·19 | 7·4 | 109 | 7·3 | 3·7 | 3·6 | 3 | 15 | — | " " " |
| 47 | 66 | — | — | B | Ad | 1·73 | 8·7 | 83 | 6·9 | 3·0 | 3·9 | 21 | 18 | 34 | |
| 48 | 68 | — | — | B | Ad | 1·48 | 11·3 | 92 | 7·4 | 4·5 | 2·9 | 3 | 9 | — | Chest infection. |
| 49 | 71 | — | — | B | Ad | 1·38 | 9·8 | 152 | 6·5 | 4·0 | 2·5 | 12 | 15 | 36 | — |
| 50 | 53 | — | Pelvis | Cl, R | — | 1·48 | 8·2 | 53 | 6·6 | 3·4 | 3·2 | 2 | 6 | 2 | After stilboestrol treatment, chest infection. |
| 51 | 68 | — | Spine | Cl, R | — | 1·85 | 6·3 | 111 | 5·7 | 3·7 | 2·0 | 33 | 25 | — | — |
| 52 | 76 | — | Chest, pelvis, spine | Cl, R | — | 1·79 | 7·6 | 195 | 5·5 | 3·6 | 1·9 | 101 | 88 | 33 | Bleeding duodenal ulcer. |
| 53a | 62 | 19.xii.49 | Pelvis | Cl, R | — | 1·73 | 8·6 | 85 | 6·3 | 4·3 | 2·0 | 12 | 58 | — | After stilboestrol treatment. |
| 53b | — | 6.ii.50 | — | — | — | 1·98 | 7·9 | 113 | 6·1 | 4·0 | 2·1 | 11 | 70 | — | — |
| 54a | 73 | 9.xii.49 | Pelvis, ribs, spine | Cl, R | — | 1·80 | 7·5 | 125 | 7·2 | 4·6 | 2·6 | 63 | 53 | — | Stilboestrol treatment from 4.xii.49. |
| 54b | — | 16.1.50 | — | — | — | 1·55 | 5·8 | 55 | 6·2 | 4·2 | 2·0 | 17 | 74 | — | — |
| 55 | 81 | — | — | B | — | 2·10 | 6·0 | 194 | 6·0 | 3·1 | 2·9 | 6 | 60 | 62 | — |
| 56 | 68 | — | — | Cl | — | 1·65 | 7·3 | 61 | 6·6 | 3·7 | 2·9 | 1 | 4 | 8 | After stilboestrol treatment. |

| No. | Age. | Date. | Proof. | LCCP (g./100 ml.). | IAI (units). | P (mg./100 ml.). | Protein. T (g./100 ml.). | Protein. A (g./100 ml.). | Protein. G (g./100 ml.). | E.S.R. (mm./hour). | Notes. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| (b) *Benign prostatic hypertrophy.* | | | | | | | | | | | |
| 63 | 54 | — | Cl | 1·60 | 11·8 | 56 | 7·1 | 4·3 | 2·8 | 3 | Urine infection. |
| 64 | 76 | — | B | 1·19 | 9·7 | 82 | 6·8 | 3·9 | 2·9 | — | Bronchitis. |
| 65 | 63 | — | B | 1·40 | 9·4 | 44 | 7·0 | 4·1 | 2·9 | — | — |
| 66 | 78 | — | B | 1·35 | 12·0 | 130 | 6·0 | 4·2 | 2·8 | — | — |
| 67 | 89 | — | B, N | 1·74 | 6·2 | 138 | 5·8 | 3·2 | 2·6 | 62 | Bronchitis. |
| 68 | 75 | — | B | 2·74 | 5·8 | 267 | 7·3 | 3·7 | 3·6 | — | — |
| 69 | 62 | — | B | 1·63 | 10·8 | 56 | 6·5 | 4·2 | 2·3 | 8 | — |
| 70 | 66 | — | B | 1·24 | 10·6 | 48 | 6·2 | 4·0 | 2·2 | 2 | — |
| 71 | 74 | — | B | 2·18 | 6·3 | 274 | 6·7 | 3·9 | 2·8 | — | — |
| 72 | 75 | — | B | 1·53 | 8·5 | 104 | 6·1 | 4·0 | 2·1 | 11 | — |
| 73 | 69 | — | N | 1·85 | 9·7 | 134 | 7·4 | 4·5 | 2·9 | — | Pneumonia. |
| 74a | 76 | 1.ii.50 | B | 2·03 | 6·2 | 151 | 5·8 | 3·4 | 2·4 | — | Cystotomy 30.i.50. |
| 74b | — | 22.ii.50 | — | 2·10 | 5·3 | 99 | 5·6 | 3·3 | 2·3 | — | Prostatectomy 17.ii.50. |
| 75 | 81 | — | Cl | 2·10 | 4·3 | 151 | 5·6 | 3·1 | 2·5 | 48 | Bronchopneumonia. |
| 76 | 83 | — | Cl | 1·98 | 4·9 | 147 | 6·1 | 3·9 | 2·2 | — | Cystitis. |

The acid and alkaline phosphatases on all these patients were normal. (For symbols see p. 385.)

## TABLE VII.—*Diseases of the Biliary Tract.*

| No. | Age. | Sex. | Primary. | Metastases. | Proof. | Histology. | LCCP (g./100 ml.). | IAI (units). | P (mg./100 ml.). | Protein. T (g./100 ml.). | Protein. A (g./100 ml.). | Protein. G (g./100 ml.). | Alk. phos. (units/100 ml.). | Bilirubin (mg./100 ml.). | Flocc. tests. | E.S.R. (mm./hour). | Notes. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (a) *Malignant obstructive jaundice.* | | | | | | | | | | | | | | | | | |
| 89 | 70 | F. | Common bile duct | — | B | Ad | 1·96 | 7·5 | 189 | 5·6 | 4·0 | 1·6 | 70 | 14 | 0 | 75 | — |
| 90 | 73 | M. | Gall bladder | — | B | Ad 3 | 2·08 | 10·3 | 167 | 6·4 | 3·0 | 3·4 | 81 | 18 | ± | — | — |
| 91 | 70 | M. | Hepatic duct | — | B, N | Ad | 1·82 | 8·5 | 91 | 5·6 | 3·1 | 2·5 | 49 | 38 | ± | — | Melaena. |
| 128 | 70 | M. | Head of pancreas | Liver | L | — | 1·81 | 7·3 | 89 | 6·6 | 3·8 | 2·8 | 26 | 13 | 0 | 28 | — |
| 129 | 49 | F. | Pancreas | Porta hepatis | L | — | 1·75 | 6·7 | 59 | 5·4 | 3·7 | 1·7 | 31 | 18 | 0 | — | — |
| 130 | 55 | M. | Liver | — | Cl, R | — | 2·08 | 7·5 | 165 | 6·4 | 3·4 | 3·0 | 104 | 4 | 0 | 5 | — |
| (b) *Infective hepatitis.* | | | | Date. | | | | | | | | | | | | | |
| 77a | 52 | M. | | 25.i.50 | | | 1·44 | 6·6 | 51 | 6·4 | 3·7 | 2·7 | 20 | 5·2 | + | — | — |
| 77b | — | — | | 30.i.50 | | | 1·40 | 8·7 | 76 | 6·2 | 3·7 | 2·5 | — | 5·0 | ++ | — | — |
| 77c | — | — | | 3.ii.50 | | | 1·50 | 9·0 | 53 | 6·0 | 3·7 | 2·3 | — | 2·8 | + | — | — |
| 77d | — | — | | 7.ii.50 | | | 1·35 | 10·0 | 56 | 6·0 | 3·6 | 2·4 | 18 | 1·8 | + | — | — |
| 77e | — | — | | 22.ii.50 | | | 1·53 | 10·6 | 55 | 6·8 | 4·1 | 2·7 | 16 | 0·9 | + | — | — |
| 78a | 20 | F. | | 5.xii.49 | | | 1·84 | 8·1 | 75 | 6·7 | 3·5 | 3·2 | 12 | 34 | +++ | — | — |
| 78b | — | — | | 8.xii.49 | | | 1·60 | 6·6 | 62 | 6·4 | 3·5 | 2·9 | — | 24 | ++++ | — | Anaemia. |
| 78c | — | — | | 12.xii.49 | | | 1·80 | 7·0 | 105 | 6·0 | 3·3 | 2·7 | — | 16 | ++++ | — | Pyrexia. |
| 78d | — | — | | 16.xii.49 | | | 1·76 | 6·7 | 114 | 5·4 | 3·2 | 2·2 | — | 22 | ++++ | — | Transfused. |
| 78e | — | — | | 21.xii.49 | | | 1·65 | 5·1 | 118 | 4·7 | 2·7 | 2·0 | — | 32 | +++ | — | — |
| 79a | 21 | M. | | 13.ii.50 | | | 1·60 | 11·8 | 107 | 7·1 | 3·5 | 3·6 | 4 | 1·6 | 0 | — | — |
| 79b | — | — | | 20.ii.50 | | | 1·32 | 11·2 | 68 | 7·5 | 4·6 | 2·9 | — | 0·8 | 0 | — | — |
| 80 | 26 | F. | | — | | | 1·20 | 12·0 | 65 | 6·0 | 4·1 | 1·9 | 8 | 7·6 | + | — | — |
| 81 | 26 | F. | | — | | | 1·44 | 12·2 | 57 | 6·4 | 4·0 | 2·4 | — | 25 | ++ | — | — |
| 82 | 32 | F. | | — | | | 1·43 | 10·4 | 58 | 6·3 | 4·0 | 2·3 | — | 2·5 | ++ | — | — |
| (c) *Hepatic cirrhosis.* | | | | | | | | | | | | | | | | | |
| 83a | 56 | F. | | 30.i.50 | | | 1·73 | 9·5 | 91 | 6·3 | 3·1 | 3·2 | 68 | 0·6 | + | — | — |
| 83b | — | — | | 4.v.50 | | | 1·65 | 9·1 | 72 | 6·6 | 3·5 | 3·1 | 58 | 1·4 | + | 53 | — |
| 84 | 54 | M. | | — | | | 2·37 | 9·1 | 61 | 7·9 | 2·8 | 5·1 | 48 | 3·2 | ++ | — | — |
| 85 | 69 | M. | | — | | | 2·44 | 9·8 | 49 | 6·1 | 2·1 | 4·0 | 23 | 0·5 | ++ | 21 | — |
| 86 | 53 | M. | | — | | | 1·62 | 6·1 | 65 | 5·9 | 2·7 | 3·2 | 11 | 6·5 | + | — | Haematemesis, melaena. |
| 87 | 59 | M. | | — | | | 2·68 | 12·4 | 48 | 6·3 | 2·4 | 3·9 | 12 | 5·2 | + | — | Accute infection. |
| 88 | 48 | M. | | — | | | 1·95 | 7·0 | 53 | 6·0 | 2·4 | 3·6 | 23 | 2·4 | + | 16 | — |

(For symbols see p. 385.)

TABLE VIII.—*Miscellaneous Diseases.*

| No. | Age. | Sex. | Date. | Primary. | Metastases. | Proof. | Histology. | LCCP (g./100 ml.). | IAI (units). | P (mg./100 ml.). | Protein. T (g./100 ml.). | Protein. A (g./100 ml.). | Protein. G (g./100 ml.). | Alk. phos. (units/100 ml.). | E.S.R. (mm./hour). | Notes. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (a) *Malignant.* | | | | | | | | | | | | | | | | |
| 92a | 60 | F. | 16.1.50 | Breast | Lymph nodes | B | D | 1·44 | 11·2 | 85 | 6·4 | 3·5 | 2·9 | 5 | — | Mastectomy 19.1.50. Bronchitis. |
| 92b | — | — | 30.1.50 | — | — | — | — | 2·02 | 6·8 | 175 | 6·2 | 3·9 | 2·3 | — | — | — |
| 93 | 71 | M. | — | Lip | Lymph nodes | B | S 2 | 1·82 | 4·5 | 102 | 6·6 | 3·7 | 2·9 | 9 | — | — |
| 94 | 80 | M. | — | ,, | — | B | S 1–2 | 1·55 | 8·7 | 55 | 6·2 | 3·9 | 2·3 | — | — | — |
| 95 | 74 | M. | — | Penis | — | B | S 2 | 1·46 | 7·4 | 89 | 7·3 | 4·2 | 3·1 | 9 | — | — |
| 96 | 46 | M. | — | Liver | — | B | Y | 1·92 | 6·1 | 181 | 5·9 | 3·6 | 2·3 | 19 | — | Laparotomy 1 week previously. |
| 97 | 38 | F. | — | Cervix | — | B | S 3 | 1·88 | 6·2 | 176 | 5·8 | 3·9 | 1·9 | 12 | 40 | — |
| 98 | 33 | F. | — | — | Bronchus | H, R | — | 2·31 | 6·7 | 240 | 7·1 | 3·8 | 3·3 | 13 | 101 | Diabetic. |
| 99 | 54 | M. | — | Myelomatosis | | N | — | 2·11 | 5·5 | 154 | 6·5 | 3·9 | 2·6 | 8 | 67 | Bronchopneumonia. |
| 100 | 76 | M. | — | ,, | | N | — | 1·83 | 8·6 | 82 | 10·4 | 3·0 | 7·4 | 8 | 123 | — |
| 101 | 50 | M. | — | Hodgkin's disease | | B | — | 1·52 | 9·8 | 98 | 6·1 | 4·3 | 1·8 | 7 | 30 | — |
| 102 | 44 | F. | — | ,, | | B | — | 1·91 | 4·0 | 135 | 4·5 | 2·8 | 1·7 | 10 | 7 | — |
| 103 | 65 | M. | — | Myeloid leukaemia | | Cl | — | 1·80 | 15·0 | 214 | 6·0 | 3·5 | 2·5 | 14 | 65 | Urethane. |
| 104 | 40 | F. | — | ,, | | Cl | — | 1·23 | 12·8 | 55 | 7·0 | 4·4 | 2·6 | 4 | 16 | X-ray therapy. |
| 105 | 70 | M. | — | ,, Aleukaemic leukaemia | ,, | Cl | — | 1·65 | 13·6 | 82 | 6·6 | 3·5 | 3·1 | 4 | — | — |
| (b) *Non-malignant.* | | | | | | | | | | | | | | | | |
| 114 | 19 | M. | Chronic nephritis | | | Cl | — | 1·45 | 9·3 | 132 | 5·8 | 3·3 | 2·5 | 14 | 70 | — |
| 115 | 23 | M. | Generalized tuberculosis | | | Cl | — | 2·56 | 5·8 | 250 | 7·3 | 3·9 | 3·4 | 14 | — | — |
| 116 | 39 | M. | Actinomycosis | | | Cl | — | 2·18 | 5·2 | 239 | 5·8 | 2·9 | 2·9 | 14 | 44 | — |
| 117 | 57 | M. | *B. coli* infection of wound | | | Cl | — | 2·40 | 5·0 | 219 | 6·0 | 2·9 | 3·1 | 12 | 76 | — |
| 118 | 22 | M. | Rheumatic fever | | | Cl | — | 1·86 | 8·7 | 202 | 6·2 | 3·8 | 2·4 | 9 | 85 | — |
| 119 | 45 | M. | Subacute rheumatism | | | Cl | — | 2·04 | 6·2 | 72 | 6·8 | 3·9 | 2·9 | — | 20 | — |
| 120 | 73 | F. | Early Paget's disease | | | Cl, R | — | 1·39 | 10·5 | 72 | 6·2 | 4·0 | 2·2 | 14 | 4 | — |
| 121 | 41 | M. | Varicose veins | | | Cl | — | 1·51 | 6·3 | 85 | 6·7 | 4·4 | 2·3 | — | — | — |
| 122 | 22 | M. | Anxiety state | | | Cl | — | 1·30 | 9·0 | 61 | 6·5 | 4·2 | 2·3 | — | 2 | — |
| 124 | 19 | M. | Hydatid cyst of liver | | | B | — | 1·52 | 8·7 | 60 | 7·6 | 4·7 | 2·9 | 11 | — | — |

(For symbols see p. 385.)

TABLE IX.—*Summary of Results.*

(First analysis on each patient only.)

| Group. | LCCP. | | IAI. | | P | | E.S.R. | |
|---|---|---|---|---|---|---|---|---|
| | + | − | + | − | + | − | + | − |
| Infective hepatitis | 2 | 4 | 1 | 5 | 1 | 5 | 0 | 0 |
| Hepatic cirrhosis | 6 | 0 | 2 | 4 | 0 | 6 | 3 | 0 |
| Benign prostate | 9 | 5 | 6 | 8 | 9 | 5 | 3 | 3 |
| Miscellaneous non-malignant | 12 | 9 | 9 | 12 | 12 | 8 | 8 | 5 |
| Carcinomatosis | 11 | 0 | 10 | 1 | 11 | 0 | 2 | 0 |
| Carcinoma of bronchus | 12 | 1 | 10 | 3 | 12 | 1 | 9 | 0 |
| „ oesophagus | 2 | 1 | 1 | 2 | 3 | 0 | 0 | 0 |
| „ prostate | 10 | 4 | 5 | 9 | 8 | 6 | 6 | 3 |
| „ bladder | 5 | 1 | 3 | 3 | 4 | 2 | 2 | 1 |
| „ stomach | 4 | 1 | 4 | 1 | 4 | 1 | 2 | 2 |
| „ colon | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 0 |
| „ rectum | 4 | 1 | 3 | 2 | 3 | 2 | 1 | 2 |
| Jaundice due to malignant growth | 6 | 0 | 1 | 5 | 3 | 3 | 2 | 1 |
| Miscellaneous malignant | 9 | 5 | 6 | 8 | 8 | 6 | 7 | 1 |
| TOTAL NON-MALIGNANT | 29 | 18 | 18 | 29 | 22 | 24 | 14 | 8 |
| „ MALIGNANT | 67 | 15 | 45 | 37 | 60 | 22 | 33 | 10 |
| % MALIGNANT CORRECT +ve | 82 | — | 55 | — | 73 | — | 77 | — |
| „ NON MALIGNANT „ −ve | — | 38 | — | 62 | — | 52 | — | 36 |

(For symbols see p. 385.)

The total protein, albumin and globulin content of sera from malignant and non-malignant cases were not significantly different. There was no obvious correlation between the serum protein concentrations and results of heat coagulation, mucoprotein or E.S.R. tests, but there was a higher proportion of positive IAI tests in sera from all cases with a low total protein content. This is illustrated in Table X.

TABLE X.—*Relationship between Iodoacetate Index and Total Protein Concentration (all cases).*

| Total protein (g./100 ml.). | No. of cases. | % +ve IAI test. |
|---|---|---|
| <5·5 | 8 | 100 |
| 5·5–5·9 | 30 | 70 |
| 6·0–6·4 | 37 | 41 |
| 6·5–6·9 | 28 | 39 |
| 7·0–7·4 | 21 | 38 |
| >7·4 | 5 | 0 |

An increase in the alkaline phosphatase was usually associated with positive "cancer tests," but no single test appears to be directly correlated with this enzyme. The serum alkaline phosphatase was raised in cases of carcinoma of the bronchus (Table IIIa, No. 13, 17, 18, 19, 20 21), stomach (Table IVa, No. 32), colon (Table IVb, No. 34), rectum (Table IVc, No. 38, 39 and 41), prostate (Table VIa), and biliary tract (Table VII).

The serum acid and alkaline phosphatase concentrations of all patients with benign prostatic hypertrophy were normal, but 10 of the 14 cases with carcinoma of the prostate showed a raised acid phosphatase, and 10 a raised alkaline phosphatase. These raised phosphatase levels are useful in indicating the presence

of secondary bone deposits, but like the other tests, they are not of much value in detecting an early carcinoma of the prostate.

The E.S.R. was raised in 80 to 90 per cent of cases (malignant and non-malignant) which gave one or more positive results in the three "cancer tests," but it was raised in 3 cases (Table IIIa, No. 15; Table IVb, No. 33; Table VIIIa, No. 104) which gave negative results. Patients with a normal E.S.R., however, did not necessarily give normal results in these tests.

## DISCUSSION.

The results of this investigation show some of the weaknesses of biochemical tests that have been advocated as diagnostic aids in neoplasia. They agree substantially with others which appeared during the present work (Huggins, Miller and Jensen, 1949; Huggins, Cleveland and Jensen, 1950; Jackson, 1950; Pollak and Leonard, 1950; Duboff, 1950; Bodansky and McInnes, 1950; Homberger, Pfeiffer, Page, Rizzoni and Benotti, 1950; Winzler and Smyth, 1948; Kelley, Good and McQuarrie, 1950; Gilligan, Rothwell and Warren, 1950; Finnegan, Brockland, Meuther, Hawk, Inkley and Thoma, 1950). The heat coagulation, iodoacetate and mucoprotein tests, which were studied in this work, are not sufficiently sensitive to detect incipient tumours, are not sufficiently reliable to exclude or to confirm a diagnosis, and generally speaking, their response is not proportional to the severity of the disease. In a variety of diseases they give false positive results. For instance, in the present series false positive results were obtained in bronchitis, cystitis, infective hepatitis, cirrhosis and actinomycosis. Of all the tests, the acid phosphatase activity of the serum appears the most useful in that it helps in the diagnosis of carcinoma of the prostate with metastases.

In various diseases the three tests gave results which conformed to a general pattern, but they differed in their sensitivity towards the change in the serum. Usually they acted similarly, giving either positive or negative results. This behaviour was not invariable, for in some diseases such as cirrhosis, positive heat coagulation tests occurred with normal mucoprotein concentration. It is, therefore, possible that the tests measure different changes which usually occur at the same time or different aspects of one change.

In carcinomatosis, the thermal coagulation (LCCP), the iodoacetate test (IAI) and the mucoprotein test (P) were consistently positive, but their degree of positivity appeared unrelated to the extent of the malignant invasion. For instance, in Case 8 (Table II), of which massive metastases were a feature, LCCP was only slightly abnormal and IAI borderline. There is usually little doubt about the diagnosis of carcinomatosis, but the detection of a single neoplasm is more difficult and urgent. In cases of carcinoma of bronchus (Table IIIa, No. 15), carcinoma of stomach (Table IVa, No. 31), carcinoma of colon (Table IVb, No. 33), carcinoma of rectum (Table IVc, No. 39), carcinoma of prostate (Table VIa, No.48, 50), carcinoma of breast (Table VIIIa, No. 92), epithelioma of lip (Table VIIIa, No. 94) and carcinoma of penis (Table VIIIa, No. 95), all three tests have given completely negative results. This failure of the tests shows up their limitations, and suggests that neither one test nor a combination of them would detect with any certainty neoplasia in an early stage of its growth.

It would seem that serum undergoes changes during an infection somewhat similar to those in malignant disease. For in most of our cases of non-malignant

disease infection was a feature, and in the majority of them the three tests gave positive results. The positivity of the tests bore no relation to the severity of the infection, so that it was impossible to judge the contribution infection might be making to any result on a malignant case. In this respect flocculation tests and white cell counts gave no help. It is therefore not surprising that the tests did not differentiate between carcinoma of the bronchus and chronic chest infections. However, it will be observed that whereas the value of the serum alkaline phosphatase was within the normal range in the non-malignant group, it was abnormal in 6 of the 13 cases of carcinoma of bronchus.

No biochemical test has so far been suggested for differentiating benign hyperplasia and malignant disease of the prostate, although the serum acid phosphatase determination is of value in establishing the diagnosis of secondary metastases. In this survey (Table VIa, b) the heat coagulation and iodoacetate tests and the estimation of mucoproteins gave normal and abnormal results in both groups. In view of the effect of infections upon these tests it would be expected that positive results would occur in the non-malignant group, since many of these elderly men had chronic infections as well as enlarged prostates. The serum proteins were of no practical value in differentiating the two conditions, but the serum acid phosphatase was raised in 10 of the 14 cases of carcinoma of the prostate and in none of the cases of benign hyperplasia. The E.S.R. was increased in 6 of the 9 cases of malignant prostate and in 3 of the cases of benign hyperplasia. It would appear that the serum acid phosphatase remains the most useful of the tests used in this survey in establshing the diagnosis of metastases, although it is of little value in the detection of early carcinoma of the prostate.

In the experience of the authors (Higgins and O'Brien, unpublished data) a small proportion of cases of infective hepatitis and cirrhosis give results which do not fit in with the general biochemical picture, and occasional sera from cases of obstructive jaundice show alkaline phosphatase levels which confuse rather than clarify the diagnosis ; such a case is No. 128 (Table VIIa). In these cases weight is given to the flocculation tests, but even these often give borderline results, especially in cases of obstructive jaundice of long duration. It was in the hope of aiding the diagnosis of these cases that the heat coagulation, iodoacetate and mucoprotein tests were done in 6 cases of infective hepatitis, 6 of cirrhosis and 6 of malignant obstruction. The results have not added to the certainty of diagnosis. The heat coagulation test was positive in all cases of malignancy, but was also positive in all 6 cases of cirrhosis and in 2 cases of infective hepatitis. When sera from some cases of cirrhosis were heated the resulting gels were turbid and consisted of two phases—a solid protein " curd " and water. These solutions were similar to those produced by Jensen, Hospelhorn, Tapley and Huggins (1950) on heating albumin in acid solution. These gels were quite different from those obtained from normal sera, and the heat coagulation and iodoacetate tests have, therefore, little meaning with these solutions. The mucoproteins show some unexpected results in that positive results were obtained in one very serious case and one very mild case of hepatitis (Table VIIb, No. 78, 79), negative results in the cirrhosis group, and variable results in the malignant group.

Physical, chemical, enzymatic and immunological investigations have shown the diversity of change in relative proportions and in their nature which plasma proteins undergo in disease. Even more subtle changes may yet be revealed as methods become more delicate and specific. How far these changes represent

a general response of the body to disease or one peculiar to one disease, is still to be decided. Of the tests which have been described, the positive response of the iodoacetate test has been attributed to changes in the molecules of protein produced by the body in repsonse to stress of illness (Bodansky and McInnes, 1950). Another suggestion is that the iodoacetate test provides an index of tissue destruction or some form of trauma. These views may be applied to other "cancer tests," and if true would rob them of real value as diagnostic aids in cancer. If some general metabolic change is responsible for positive results in these tests, it is not surprising that abnormal results are found in diseases other than cancer. Nor is it surprising that some sera from cancer patients give normal results, since it is difficult to understand how some isolated neoplasm, such as epithelioma of the lip, could affect protein metabolism.

Of the changes in plasma proteins in disease and of many of the reactions used to reveal them there is still much to be learned. The deficiency in thermal coagulation, as shown by the heat test, may be due to an alteration in the albumin molecule, which is possibly associated with a change in the number of free sulphydryl groups. No gross change in the amino-acid composition of the albumin in these sera has been detected (Huggins, Jensen, Player and Hospelhorn, 1949). Heat denaturation of protein may, however, involve a breaking of hydrogen bonds which hold the protein molecule in its natural form, thus causing the alteration in its configuration and the formation of new hydrogen bonds between carboxyl and amino groups in neighbouring polypeptide chains (Mirsky and Pauling, 1936). Possibly the protein molecule, produced by the body in response to, or because of, the disease, differs from the normal protein in its arrangement of the amino-acid groups, resulting in a different molecular pattern and a differing availability of the carboxyl and amino groups. Jensen, Hospelhorn, Tapley and Huggins (1950) have suggested that iodoacetate reacts with the sulphydryl groups in the albumin molecule, thus introducing a negatively charged carboxyl ion which prevents lateral binding of protein molecules on heating, and that this inhibits coagulation. The decreased amount of iodoacetate necessary to prevent coagulation of sera from diseased patients may be associated with a decreased number of sulphydryl groups. This theory is supported by the report of Schoenbach, Weissman and Armistead (1951) that the sulphydryl content of serum protein from some patients with malignant and other diseases is subnormal. However, as shown in Table XI, a decreased serum protein concentration is usually associated with a positive iodoacetate test. Similar effects were noted by Huggins, Miller and Jensen (1949) and Duboff (1950). It would therefore appear that the reaction is not a simple combination with sulphydryl groups. It is possible that electrostatic forces in the protein-depleted serum are altered so that less iodoacetate is required to bring about a sufficiently negative charge to hinder coagulation. With the elucidation of points such as these, the value of biochemical tests in diagnosis may eventually be properly assessed.

Whatever may be the nature of the change in the protein molecule responsible for the abnormal results of heat coagulation, iodoacetate and mucoprotein tests, it occurs in malignant and non-malignant disease to an extent often disproportionate to the severity of the disease. The tests like the E.S.R. indicate an abnormality of unspecific and as yet undefined nature. Added to this weakness, they often give negative results in cases of neoplasia so that they have little value as "screening tests." Of the other determinations made in this investigation, the

serum protein concentration is of little value in the differential diagnosis of malignant disease, and in many cases no guide to the severity of the condition. The acid phosphatase is of value in the diagnosis of carcinoma of the prostate with metastases, and the alkaline phosphatase a useful aid in the diagnosis of liver or bone disease.

In future work it is hoped to investigate more closely the nature of the change in the plasma protein in malignant disease and to correlate it with biochemical studies upon biopsy specimens.

SUMMARY.

1. The heat coagulation and iodoacetate tests and estimation of mucoproteins have been performed, together with other biochemical tests, upon 25 normal subjects, 82 patients with neoplasia and 47 patients with non-malignant diseases.

2. The heat coagulation test (LCCP) was positive in 82 per cent of carcinoma cases and 62 per cent of non-malignant cases.

3. The iodoacetate test (IAI) was positive in 55 per cent of carcinoma cases and 38 per cent of non-malignant cases.

4. The mucoprotein concentration (P) was increased in 73 per cent of carcinoma cases and 48 per cent of non-malignant cases.

5. No correlation was found between the degree of positivity of these tests and histology or extent of the carcinoma. The iodoacetate test, however, appeared to be related to the serum protein concentration.

6. This survey shows that these tests add little to the laboratory diagnosis of cancer.

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# RADIOACTIVE METALLIC GOLD COLLOIDS COATED WITH SILVER AND THEIR DISTRIBUTION IN THE LUNG AND ITS LYMPHATICS FOLLOWING INTRA-PULMONARY ADMINISTRATION: THERAPEUTIC IMPLICATIONS IN PRIMARY LUNG AND BRONCHIOGENIC TUMORS.

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BRONCHIOGENIC carcinoma, a comparative rarity a generation ago, is not only six times more commonly diagnosed than 25 years ago, but is considered by some authorities to be the most frequently encountered malignant tumor in males to-day. Graham (1950) has stated that only about 25 per cent of such cases are candidates for total pneumonectomy. Others claim that the overall cure rate by surgery, with or without accompanying x-irradiation, is only about 5 per cent. Apparently late diagnosis, due to paucity of symptoms at an early stage and inadequacy of diagnostic methods, are largely responsible for the number of cases which are not subject to present methods of treatment. Therefore any possible means of attack in this disease condition warrants careful appraisal and exploitation.

It has been demonstrated by Meneely, Quarles and Curtis (1951) that when water-soluble materials tagged with radioactive isotopes are administered by tracheal route, the water is rapidly absorbed by the alveoli of the lungs. When radioactive metallic colloidal gold sols were administered under similar circumstances it was found that the fluid vehicle was absorbed, and the gold remained for many days in the lung parenchyma and did not gain access to the blood stream. In these latter experiments, aimed at not only the distribution of the gold colloid, but also at determining the tolerance of the animal to withstand irradiation of selected portions of the lung, large doses were administered at therapeutic levels. However, the gold colloid was drained very slowly by the regional lymphatics, it taking from 10 days to 2 weeks in order to attain concentrations sufficient to permit adequate radiation of these tissues which play an important part in the usual metastatic sequelae in bronchiogenic carcinoma. The gold isotopes $Au^{198}$ and $Au^{199}$ having half-lives of 2·7 and 3·3 days respectively would thus normally be unsuitable for therapeutic use in this disease, at least when used alone.

Therefore other possible isotopes were considered for experimental use, and it was thought that $Ag^{111}$, with a half-life of 7·5 days, might offer possible advantages, especially since it meets most of the criteria for use as a therapeutic isotope (Hahn, 1951). Upon trial it was found that the colloids of metallic silver were rapidly transported to the regional lymphatics of the lung following intra-pulmonary administration. Unfortunately this isotope of silver is difficult to produce in the

pile by reaction of thermal neutrons on palladium, since the cross-section is none too good and the parent palladium isotope is not highly abundant. Therefore the economy of production leaves much to be desired at the present time. As a result we decided to attempt to produce non-active silver-coated radioactive gold colloids, hoping the body would show a tendency to distribute this latter material according to the chemical and physiological reactivity of silver. Still, we should be able to utilise the radiation from $Au^{198}$, whose half-life is such as to allow a better " titration " of the patient response, and to take advantage of the other favorable characteristics of this very useful isotope (Hahn, 1951). As shown below, it was readily found that the body was unable to discriminate between such a coated gold colloid and a silver colloid in the time necessary for the desired distribution to occur.

It was not possible to employ therapeutic doses in the present experiments, due to the relatively low specific activities of the gold isotope used for seeding the dispersion of the silver colloid.

## METHODS.

The silver-coated gold colloids were prepared as follows: To a solution containing 50 mg. of silver in the form of the nitrate was added a fraction of a ml. of metallic colloid of $Au^{198}$, which had been prepared by neutron bombardment of $Au^{197}$ in the Oak Ridge, Tennessee, pile and subsequently processed as a sterile pyrogen-free colloid by Abbott Laboratories, North Chicago, Illinois, and which already contained an excess of ascorbic acid. This resulted in the immediate dispersal of some of the silver. Three drops of 40 per cent NaOH and 1 ml. of 6 per cent gelatine (as a protective colloid) were added, followed by the addition of a solution of cevitamic acid containing 250 mg. of the latter reducing agent. The resultant dispersion appeared grayish-yellow to reflected light and a slate color with a yellow tinge to transmitted light. Water was added to make up the total amount of fluid desired, the latter being calculated such that 0·5 ml./kg. of body-weight was to be administered to each animal as a tracer dose. An aliquot of 1 ml. of the sol as administered was reserved for subsequent radioactivity measurements.

The dogs used in these experiments were coon-hounds of about 18 months of age which had been de-wormed, vaccinated against distemper and maintained on a diet of " Purina Chow " for several months prior to use. They were anesthetised with nembutal (35 mg./kg.) and a small-sized bronchoscope inserted into the lower middle right lobe of the lung. At this juncture a copper inner tube (refrigerator tubing) of small outside diameter, to which had been soldered the hub of a needle, was inserted into the bronchoscope, and the colloid in a lock-type of syringe was attached and the material injected. About 1 ml. of air was then injected in order to empty the inner tube and the bronchoscope was withdrawn. The animals were then laid on their right sides, and the upper end of the table was raised and they were left until recovery of the anesthesia was nearly complete.

The dogs were sacrificed at intervals of 2, 5 and 10 days and samples of tissues taken for histological examination and radioactivity measurements. In carrying out the autopsy the abdomen was opened first and specimens taken of liver, spleen, kidney and mesenteric lymph nodes, all of which would be expected to

show low levels of radioactivity. When the thorax was opened every effort was made to avoid contamination from tissues having higher levels of radioactivity, this being accomplished by taking tissues from the left side of the lung first and subsequently taking lymph nodes and pulmonary tissue from the right side.

Radioactivity was determined on weighed samples of fresh tissues by means of a thin mica window end-window type of Geiger-Müller tube in conjunction with a scaling circuit. For the purposes of this study it was not necessary to ash the tissue samples for such measurements. The latter step is necessary, however, in using the $Ag^{111}$ isotope.

TABLE I.—*Distribution of Gold Colloid Nucleus as Measured by Tissue Radioactivity.*

| | 51–1. 2 days. | | 51–12. 2 days. | | 51–10. 5 days. | | 51–18. 10 days. | |
|---|---|---|---|---|---|---|---|---|
| | cpm./g. | %. | cpm./g. | %. | cpm./g. | %. | cpm./g. | %. |
| Right lung—treated lobe | 230,000 | 100 | 1,550,000 | 100 | 171,000 | 100 | 142,000 | 100 |
| ,, ,, other lobe | 51,000 | 22 | 609,000 | 40 | — | — | 75,000 | 53 |
| Left lung | 7,600 | 3·3 | 19,800 | 1·3 | 10,000 | 5·8 | 16,800 | 12 |
| Bifurcation lymph node | 47,800 | 21 | 338,000 | 22 | 118,000 | 69 | 238,000 | 167 |
| L. tracheal ,, | — | — | 55,200 | 3·6 | 48,000 | 28 | — | — |
| R. tracheal ,, | — | — | — | — | 3,900 | 2·3 | 148,000 | 104 |
| R. bronchial ,, | — | — | 11,700 | 0·8 | — | — | 446,000 | 314 |
| L. mediastinal ,, | 1,320 | 0·6 | 18,500 | 1·2 | 32,000 | 19 | 187,000 | 131 |
| R. mediastinal ,, | 40,300 | 18 | 72,800 | 4·7 | 54,000 | 32 | 93,000 | 65 |
| L. hilar ,, | 77,200 | 34 | — | — | — | — | — | — |
| R. hilar ,, | 77,100 | 34 | — | — | — | — | — | — |
| Mesenteric ,, | — | — | 39 | 0·002 | 136 | 0·1 | 193 | 0·1 |
| Thoracic ,, | — | — | — | — | — | — | 150 | 0·1 |
| Kidney | 272 | 0·1 | — | — | 507 | 0·3 | — | — |
| Liver | 180 | 0·08 | 980 | 0·06 | 2,610 | 1·5 | 1,560 | 1·1 |
| Spleen | 33 | 0·01 | 248 | 0·02 | 363 | 0·2 | 501 | 0·4 |

cpm./g. = arbitrary counts per minute per gram of fresh tissue measured on Geiger counter.
% = concentration of radioactivity relative to treated lobe of right lung taken as 100.

*Experimental Observations.*

In Table I is shown the distribution of the radioactive gold nucleus of the colloid introduced in 4 dogs studied. Actually experiments were carried out in Dogs 51–1 and 51–10 first, they being sacrificed at 2 and 5 days respectively. When it was noted that considerable drainage had occurred in as little as 2 days this experiment was repeated in Dog 51–12 and the results were confirmatory. It should be stated here that the inhomogeneity of the data resulted from failure to find nodes associated with various structures in many instances.

Radioactivity is expressed in arbitrary "counts per minute per gram of fresh tissue", corrections being made for the various scaling circuits used. The efficiency of our counter tube for these emanations, including consideration of geometry, is about 20 per cent, and therefore the actual number of disintegrations was about five times that shown in Table I. Since there was some discrepancy in the total amount of radioactivity of the administered material, and since the time intervals are at variance, the results are also expressed in terms of concentrations relative to that found in the lobe of the lung which was instilled.

The material was injected as rapidly as the lumen of the copper inner tube would permit with moderate pressure on the plunger of the syringe. When carried out outside the animal it resulted in a stream of fluid at the effluent end

of the tube extending 3 to 4 inches beyond the orifice. Presumably this would result in a rather turbulent type of introduction of the colloid into the lung involved and should assist in obtaining relatively uniform distribution therein.

Examination of the data shows that in the first 3 dogs there was relatively little of the material in the contra-lateral lung. Thus under the conditions of anesthesia and positioning of the animal subsequent to delivery of the material there was little aspiration or regurgitation of the fluid.

That there was a fairly considerable amount of the active material found in other lobes of the treated lung suggested that possibly the amount of fluid vehicle used was too great. Earlier work of Meneely, Quarles and Curtis (1951) and others (Meneely, Kory, Auerbach and Hahn, 1951) using water tagged with small amounts of radioactive materials and using gold colloids, respectively, showed that water in the quantities used was rapidly and efficiently absorbed. If the animal did not cough within about 10 minutes following administration of the sol there was little found elsewhere than where directed. It may be that the silver is more irritating and thus causes this difference in distribution. In fact it is possible that such irritation may well be the underlying reason why silver colloids are drained more readily by the lymphatics.

As can be seen from Table I the quantity of the gold nucleus of the colloid found in the liver and spleen was minimal, indicating that essentially none of the material obtained access to the circulation (Hahn, 1951).

Whereas when gold colloids are administered by various routes, being inert, they remain *in situ*, their biological, effective and physical half-lives are practically identical, this is not the case when we use silver or silver-coated colloids. The transient stay of these colloids discussed here requires further study before much can be suggested regarding therapeutic dosage levels. The well-tolerated dose of the straight gold colloid given by the intra-pulmonary route has been found to be about 0·25 mc. per pound of body-weight in dogs. That dosage of the silver-coated gold colloid due to its distribution between the lung parenchyma and lymph nodes will be somewhat greater is reasonable to predict.

## DISCUSSION.

In the use of gold colloids interstitially in treatment of breast tumors we have been impressed by the fact that relatively small quantities of the colloid appeared in the lymphatics draining such tissue (Hahn, 1951). It was felt that the tumor tissue partially or completely occluded the lymphatic channels in these far advanced cases. However, we see that in normal dogs, with no obvious reason for obstruction of such channels, there is relatively slow drainage by the lymphatics. The present and other experiments with silver colloids indicate that the latter and silver-coated colloids behave differently. This would therefore appear to be a property of the silver itself and the body reaction to this metal from the chemical and physiological standpoint. It would suggest that some type of silver colloids should be very useful in studies of the lymphatic drainage in the body under normal conditions as well as in pathological states. In this respect the radioactive colloids should prove far superior to india ink and dyes since quantitation is easily effected with the isotopic tracers.

The early concentration of the radioactive material in the nodes at the bifurcation of the trachea is of interest, since it occurred in every instance and in as little as 2 days to an appreciable extent. In the animal 51–10, sacrificed at 5 days,

considerably greater accumulation had taken place in most nodes. At the end of 10 days (51–18) the concentration of the colloid nucleus had in most nodes exceeded that in the lung into which it had been instilled. This is in great contrast to the relative slowness with which the straight gold colloid was drained, the latter taking this much time to appear in 50 per cent concentration in the highest active node.

It should be pointed out that in several instances, as seen in Table I, there was preferential drainage of the contra-lateral nodes. In Dog 51–10 the left tracheal node and in Dog 51–18 the left mediastinal node showed many times the concentration of the gold nucleus after 5 and 10 days respectively, in the opposite side from which it had been introduced. This seems particularly interesting in view of the frequency with which primary lesions on one side of the lung or breast are associated with spread to the contra-lateral side. We apparently know little concerning the lymphatic drainage of the body, and it is to be hoped that radioactive colloids may prove useful in extending our knowledge in this direction.

Since gold colloids remain in the lung parenchyma for long periods and since the silver colloids are quite rapidly drained, ultimate use of these agents in treatment might well consist in use of a combination of the two. The gold colloid would be useful in destroying the primary tumor bed, that lobe of the lung necessarily being considered expendable. The silver-coated gold colloids would then be employed in treating the metastatic lesions in the lymphatics draining the region. Obviously many technical details and studies of dosage tolerances remain to be determined before these potentially useful agents may be successfully used in therapy. However, the latent possibilities are apparent and deserve exploitation.

SUMMARY.

When silver is dispersed in the presence of radioactive metallic colloidal gold particles as nuclei, a colloid is formed which acts as though it were a silver colloid as regards chemical and physiological behaviour. Administered by intra-pulmonary route by means of a bronchoscope the silver-coated gold colloid is rapidly removed from the lung parenchyma in considerable proportions and appears in the lymph nodes draining the lung region.

Silver-coated $Au^{198}$ metallic colloids may offer a means of providing selective radiation to the lung parenchyma, as well as the lymph nodes draining the pulmonary system in malignant disease of the bronchus and lung.

This study was carried out under contract with the Division of Biology and Medicine of the U.S. Atomic Energy Commission.

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# AN INVASIVE RED-PIGMENTED TUMOUR (ERYTHROPHOROMA) IN A RED MALE PLATYFISH (*PLATYPOECILUS MACULATUS* VAR. *RUBRA*).

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THE corium of fish contains a variety of pigment cells—melanophores, erythrophores, xanthophores and guanophores, each capable of producing tumours. The most commonly occurring pigmented tumour is the melanoma, which has been described in several species (Takahashi, 1929; Johnstone, 1925; Gordon, 1948; Reed and Gordon, 1931; and others). Xanthophoromas and guanophoromas are rare tumours and only one case of each type has been reported (Schroeders, 1908; Takahashi, 1929).

So far only a few red-pigmented tumours have been studied. Takahashi (1929) reported a case in a gurnard (*Chelidonichthys knmu*). The primary was situated in the skin on the left side just behind the head, and had infiltrated the muscles down to the vertebrae and bulged into the coelom, but there were no metastases. Thomas (1931) has described two instances of localized red tumours, one in the subperitoneal tissues of a tunny fish (*Gymnosarda alleterata*) and another in the anal fin of a trout (species not recorded): in this case a small metastasis was found on the surface of the peritoneum.

Kosswig (1929) has described localized red tumours in two male hybrid fish obtained by crossing an $F_1$ Killifish hybrid to a pure line red-finned platyfish. Later on (Kosswig, 1931) he obtained a single case of an erythrophoroma in an $F_1$ hybrid of *Xyphophorous montezuma* and *Xyphophorous hellerii* and another in an $F_1$ hybrid of *Xyphophorous hellerii* and *Platypoecilus maculatus*. When the latter hybrid was mated to *X. hellerii* erythrophoromas were found in five offspring. However, Gordon (1937), discussing these tumours, states, "It is not clear in these cases whether the erythrophores form an erythrophoroma or the erythrophores are merely incorporated in the macromelanophoroma." Nigrelli, Jakowska and Gordon (1951) have studied 7 representative specimens from a total of 27 *X. hellerii* × *P. maculatus* hybrids with red or red-black tumours (xanthoerythrophoroma and erythromelanoma) obtained by genetic methods.

Smith (1934) described an erythrophoroma with metastases in a flat fish (*Pseudopleuronectes americanus*). The main tumour measured 3 × 2 cm., and was situated on the upper pigmented side of the fish 4 cm. behind the operculum and had penetrated into the extraperitoneal muscles; four cutaneous metastases and 23 visceral metastases were described. Smith, Coates and Strong (1936) found a brick-red tumour in the region of the dorsal fin of a *X. hellerii* × *P. maculatus* hybrid. The tumour showed local invasion but no distant metastases. The erythrophoma studied by us is the first case of such a tumour to be recorded in a pure line platyfish.

*History.*

The tumour occurred in one of 4 red male platyfish kept in a home aquarium. It was first noticed 2 months before death. It increased rapidly in size (Fig. 1, 2) without causing much inconvenience to the fish until a few days before death, when the fish was found lying at the bottom of the tank unable to surface. As death seemed imminent the fish was killed and fixed.

The breeder of the fish was interviewed and he reported that the fish came from a pure line strain of red platyfish, imported in 1946, from which he had inbred for over 6 years in this country. There is no evidence to suggest that these fish have ever been crossed with any other species of live bearer. This is further confirmed by the fact that during the prolonged period of breeding, involving numerous generations of fish, there has been no atypical progeny. Although the tumour-bearing fish (Fig. 1) has not the classical " chunky " platy form (due probably to " malignant cachexia "), other healthy specimens from this strain examined by us show the classical platy characteristics. During the course of breeding from this strain the breeder reported that he had noticed occasional occurrences of " red lumps " on some of these fish.

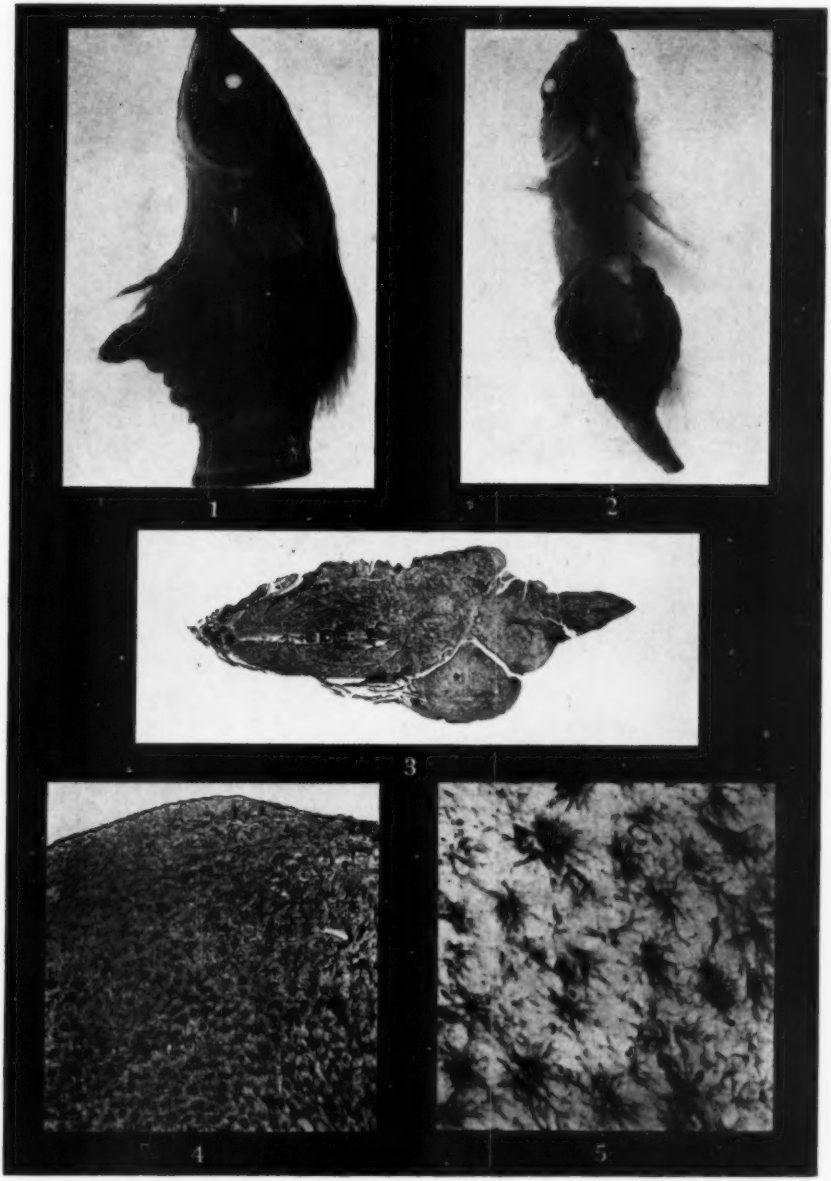
*Description of specimen.*

*Macroscopical appearance.*—The fish was an emaciated male platy aged 9 months (note scaphoid abdomen in Fig. 1) measuring approximately 2·1 cm. from the snout to the root of the caudal fin. It was of a uniform deep matt red colour and showed no melanotic spotting or stippling. The tumour measured approximately 8 mm. × 8 mm. × 5 mm., and was situated on the ventral and ventrolateral surfaces in the region of the gonapodium, which was ensheathed by the tumour mass (Fig. 1, 2). The colour of the tumour was a deep scarlet and the surface coarsely lobular. On section the tumour was a dark red firm mass which encircled the body of the fish ventrally and ventrolaterally, and extended deeply into the muscle mass (Fig. 3). No metastases were observed. The scales around and over the tumour were thickened and appeared fleshy.

*Microscopical appearance.*—The cytological elements of this tumour show considerable pleomorphism. At the periphery the tumour consisted mainly of small and large stellate cells with small nuclei and abundant vacuolated cytoplasm (Fig. 4, 5). The central parts of the growth showed a more bizarre pattern with fusiform cells, round cells, stellate cells and multinucleated forms (Fig. 6).

EXPLANATION OF PLATES.

FIG. 1.—Left lateral view of the red platyfish showing tumour mass, ensheathed gonapodium, ventral fins and scales. × 3·5.
FIG. 2.—Ventral view of same fish. Note large size of tumour. × 3·5.
FIG. 3.—Transverse section of fish at level of gonapodium showing extensive infiltration of the ventral muscle mass, gonapodium and scales. × 6.
FIG. 4.—Peripheral zone of tumour showing stellate cells. Note intact epithelium on surface of tumour. H. & E. × 84.
FIG. 5.—Stellate cells and fine pigment granules. H. & E. × 300.
FIG. 6.—Area of tumour showing multinucleate cells. H. & E. × 135.
FIG. 7.—Unstained frozen section showing birefringent material in tumour cells. × 100.
FIG. 8.—Scales embedded in tumour cells. H. & E. × 55.
FIG. 9.—Hyperplastic erythrophoretic tissue (erythrosis) distant from tumour site. H. & E. × 55.
FIG. 10.—Invasion of striated muscle. H. & E. × 270.

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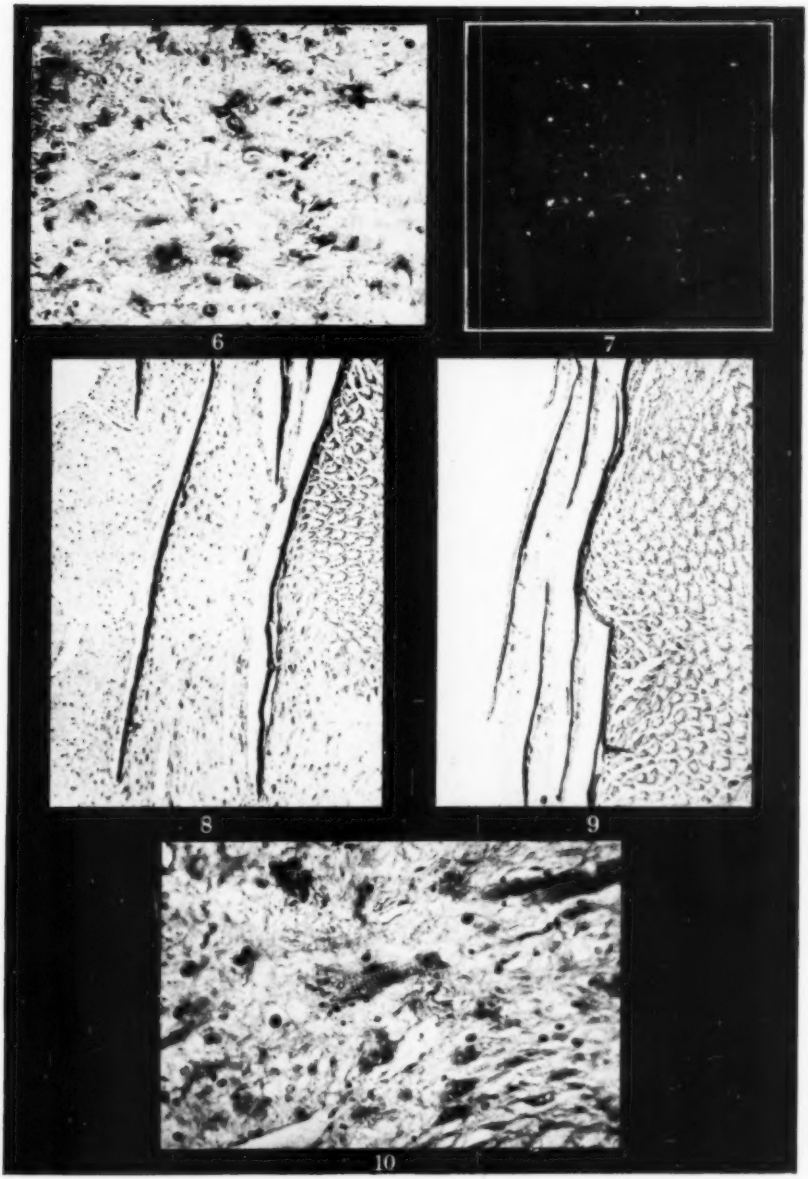

Ghadially and Whitoley.

There was also considerable variation in the size and shape of nuclei, varying from the large vesicular with chromatin granules condensed on the nuclear membrane to small dark pyknotic type with no obvious chromatin pattern. Mitotic figures were extremely scanty.

In haematoxylin and eosin sections the cytoplasm had a foamy vacuolated appearance, in many places obscuring the cell outline. Most of the cells contained a very fine granular brownish-red pigment in varying amounts. Unstained frozen sections showed numerous fine yellow to orange lipoid droplets in the cytoplasm, and these had a tendency to a perinuclear arrangement. The colour of this lipoid material faded almost completely on keeping. Birefringent material was present in many of these cells (Fig. 7).

The carnous appearance of the gonapodium, ventral fins and the scales adjacent to the tumour was found to be due to the embedding of these structures in thick sheets of tumour cells (Fig. 8). This change was also present, but to a lesser degree, in scales distant from the tumour (Fig. 9). There was extensive destructive infiltration of the underlying musculature (Fig. 3, 10), involving a large part of the ventral muscle mass.

## DISCUSSION.

Apart from the few naturally occurring erythrophoromas, the only other reported tumours have been produced by genetic methods in *P. maculatus* × *X. hellerii* hybrids (Nigrelli, Jakowska and Gordon, 1951; Smith, Coates and Strong, 1936; Kosswig, 1929, 1931). Nigrelli, Jakowska and Gordon (1951) find that xanthoerythrophoromas (tumours similar to the one described in this paper) and melanomas occur in the platyfish-swordtail hybrid due to the modifying influence of the swordtail genes on the colour-producing genes in the male ruby-throated spot-sided platyfish, and further that these tumours occur only in hybrids which have both erythrophores and macromelanophores.

However, the evidence presented in this paper shows that erythrophoromas can occur in the platyfish in the absence both of the modifying swordtail genes and melanophores, as there is no evidence to suggest that our specimen is a hybrid, nor were any spots or stipples present on this fish or other fish from this strain so far examined. Histological examination confirmed the absence of melanin-containing cells in the corium of the fish and tumour.

It will be observed (Fig. 9) that there was hyperplasia of the erythrophoretic tissue distant from the tumour mass, and that this did not show the pleomorphism of the main tumour mass, but was composed of almost normal-looking erythrophores. It is possible, therefore, that this does not represent an invasion of tumour cells in the corium of the fish, but a state of hyperplasia, possibly genetically determined. This is further supported by the fact that we have observed varying grades of hyperplasia in other tumour-free fish from this strain. It seems therefore that this tumour has arisen from a large field of genetically determined hyperplastic tissue, and enlarged not only by cellular proliferation and invasion, but also by progressive neoplastic transformation of hyperplastic tissues within these areas. Thus the genetic constitution of these fish provides susceptible, unstable pre-cancerous areas of tissue which may undergo a malignant change *in situ*. This hyperplastic state, with or without an obvious tumour, may be termed erythrosis, and is analogous to the varying grades of melanosis described by Reed and Gordon (1931) in the platyfish-swordtail hybrids.

SUMMARY.

A case of an invasive red-pigmented tumour (erythrophoroma) has been described in a pure line red platyfish (*P. maculatus* var. *rubra*). This was a rapidly growing tumour which was locally invasive but showed no metastasis. It would appear that this tumour had arisen in a pre-existing genetically-determined bed of hyperplastic tissue (erythrosis).

This is the first record of an erythrophoroma in a pure line platyfish, all others having been reported in platyfish-swordtail hybrids or other species. Thus it would seem that though the modifying swordtail genes described by Nigrelli, Jakowska and Gordon (1951) may be a factor in tumour production in the hybrid, their absence does not preclude the development of tumours in the pure line platyfish.

We would like to thank Professor H. N. Green for valuable advice and criticism. The tumour occurred in a home aquarium kept by Mr. D. J. Wallace, and we are deeply grateful to him for the opportunity of examining the specimen and for his observations on the natural history of the disease. We are indebted to the breeder, Mr. J. D. L. Fleetwood, for supplying the genetic history of the strain, and to Mr. A. W. Collins for the photomicrographs.

Since describing the above specimen a histologically identical tumour, approximately $\frac{1}{2}$ cm. in diameter, invading the muscles has developed in another one of the 4 male red platyfish.

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# MOUSE LIVER CATALASE. THE ANTAGONISTIC EFFECT OF TUMOUR TISSUE UPON THE HORMONAL CONTROL MECHANISMS.

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THE presence in tumour tissue of some substance capable of depressing the liver catalase activity in mice has been established recently in a number of laboratories (Nakahara and Fukuoka, 1949, 1950; Adams, 1950*a*, 1951; Greenfield and Meister, 1951). It therefore seems reasonable to suppose that the action of a growing tumour on liver catalase is due to the release of this substance into the circulation. So far, however, little is known of the mechanism by which the catalase-depressing substance exerts its effect, or of any significance it may have so far as the tumour itself is concerned. In a preliminary report on the changes in mouse liver catalase level which occur after castration and adrenalectomy, it was tentatively suggested that tumour tissue might be acting by interference with hormonal stimulation of the liver (Adams, 1950*b*). Before this suggestion could be investigated, it was found necessary to examine in some detail the hormonal factors influencing liver catalase activity in the normal mouse. The results obtained may be briefly summarised as follows. There is a sex difference in normal catalase activity, males having the higher level. Castration of males reduces the catalase activity to slightly below the normal female level within 48 hours. Administration of testosterone to castrated males restores their normal catalase activity; when administered to normal females it elevates their catalase level to that of the male. Adrenalectomy in both sexes produces a fall in activity, restored to normal by cortisone (Adams, 1952). Adrenalectomy in rats also results in a depression of liver catalase activity, according to Begg and Reynolds (1950).

The present paper describes some preliminary results which support the hypothesis that in an animal treated with tumour homogenates, the adrenal and testicular factors normally influencing liver catalase activity are prevented from operating. This failure appears to be due to the inability of the liver to respond to the hormonal stimuli.

## MATERIALS AND METHODS.

*Animals.*—Young adult mice of a stock albino strain (weighing 25 to 30 g.) were used. The diet of the animals consisted of rat cubes (Rowett Institute formula), and water *ad libitum*.

*Tumour.*—Sarcoma 37 (originally obtained from the Chester Beatty Research Institute) has been used throughout. The tumour was administered by the injection of a coarse homogenate prepared with a TenBroeck grinder.

*Estimation of liver catalase.*—Samples of whole liver homogenates were allowed to react with M/40 hydrogen peroxide (A.R. quality) in M/50 phosphate buffer (pH 6·8) at 0° C., the reaction being stopped after 4 minutes by the addition of sulphuric acid. The hydrogen peroxide remaining was estimated by titration with standard sodium thiosulphate solution, after the addition of excess potassium iodide. Corresponding determinations of total nitrogen were made (by Kjeldahl), catalase levels being expressed in arbitrary units/mg. N. For details see Adams (1950*a*).

*Operative procedure.*—Castration and adrenalectomy were carried out under ether-chloroform anaesthesia, as previously described (Adams, 1952). In an additional attempt to combat shock following adrenalectomy, glucose (0·5 per cent w/v) was added to the drinking water (already containing 0·5 per cent sodium chloride) for 48 hours following operation.

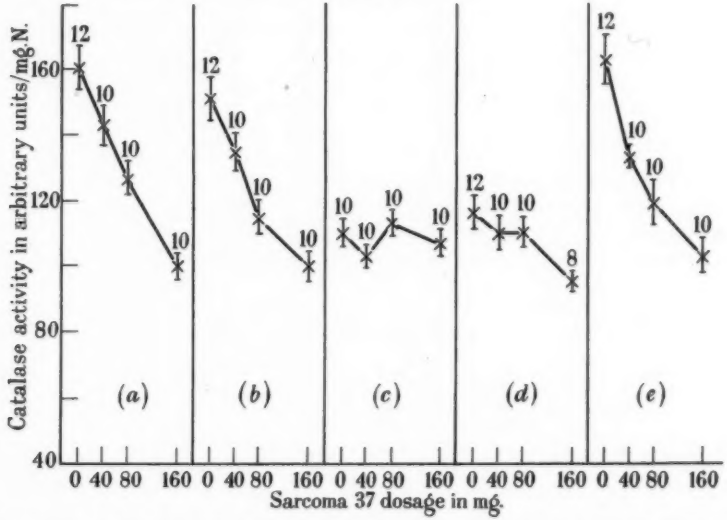


FIG. 1.—Effect of the intraperitoneal injection of varying doses of Sarcoma 37 homogenate on the liver catalase activity of the groups of mice detailed. Catalase levels 48 hours after tumour injection. (*a*) normal males, (*b*) castrated males receiving testosterone (60 μg./day), (*c*) castrated males, (*d*) normal females, (*e*) normal females receiving testosterone (60 μg./day). Results are given as arithmetic means ± standard error of means. The number of animals in the groups are given by the small figures at the head of the standard error limits.

*Hormones.*—Testosterone (commercially supplied for implantation). Cortisone (17-hydroxy 11-dehydro corticosterone), kindly provided by Professor C. Wilson. These were injected subcutaneously in 50 per cent alcohol-water solution and in daily doses.

## RESULTS.

During the coûrse of the previous investigations (Adams, 1950*a*, 1951) it was observed that, coupled with a sex difference in normal liver catalase activity (males > females), there is a bigger initial fall in male catalase level after tumour

injection. This was true of all four tumours which were injected into both sexes. To investigate this effect further, curves of Sarcoma 37 dosage against catalase level 48 hours after intraperitoneal injection were plotted for the following 5 groups of mice—normal males, castrated males, castrated males receiving testosterone (normal catalase level restored), normal females, females receiving testosterone (catalase elevated to approximately the normal male level). The groups treated with testosterone were injected subcutaneously with 60 μg./day during the experiment, the tumour homogenate being given on the sixth day. The results appear in Fig. 1, and show that normal males and castrated males

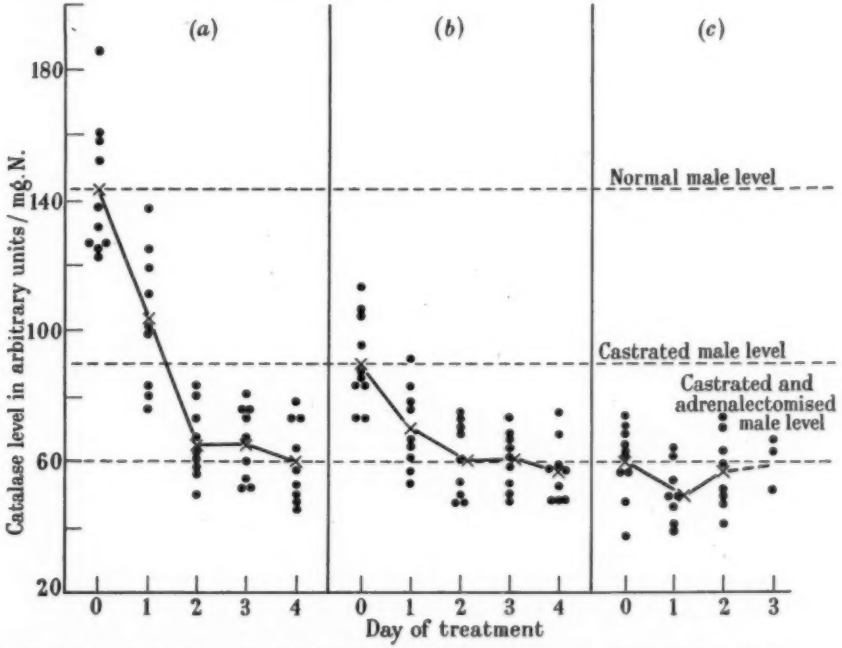


FIG. 2.—Effect of the repeated daily injection of 100 mg. S37 (as homogenate) on the liver catalase activity of (*a*) normal males, (*b*) castrated males, and (*c*) castrated and adrenalectomised males. The control groups (no S37) appear on the left-hand sides of the graphs (abscissa points 0, 0, 0). In this and the subsequent figures the points represent individual mice and the crosses the arithmetic mean values of the groups.

receiving testosterone behaved in a very similar way; there was a progressive fall in catalase activity with increasing tumour dosage, which was approximately equal in the 2 groups. The effect of the tumour on castrated males was not significant, contrasting with the steep fall in the first 2 groups. Normal females showed a small depression in catalase activity after the injection of tumour, the change in level being less than with normal males, as previously observed. The catalase depression in the females receiving testosterone, however, was closely similar to that shown by normal males and castrated males receiving testosterone.

Hitherto in this work tumour has been administered in single doses, and the

catalase activity determined after an interval or intervals. It seemed of interest to determine the maximum depression of liver catalase which can be produced by tumour homogenates in the absence of tumour growth, and this was done by observing the effect of the repeated injection of high doses. Up to about 5 daily injections may be given before visible signs of tumour growth appear. Sarcoma 37 in 100 mg. doses was injected daily into normal male mice by the intraperitoneal route, and the catalase level determined daily. Fig. 2 shows the results of this experiment. The catalase activity fell from the control level of some 140 arbitrary units/mg. N. to 65 arbitrary units/mg. N. within 48 hours, and then remained constant. It seemed significant that this is approximately the level of activity reached after castration and adrenalectomy. Fig. 2 also shows the effect of injecting 100 mg./day of S37 into castrated males. Although the initial level

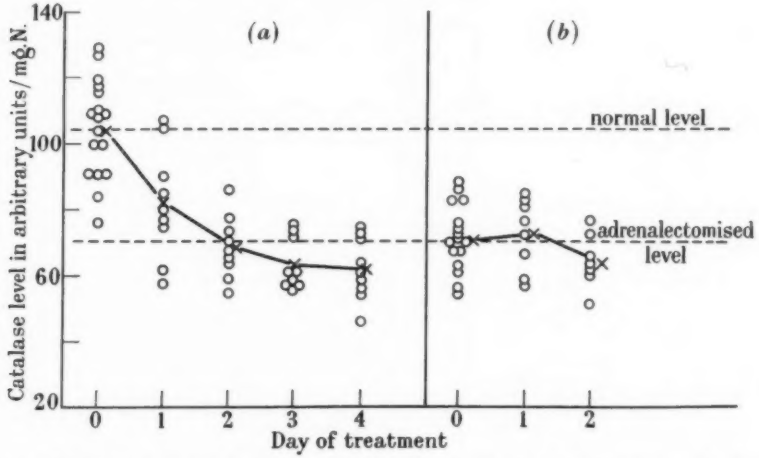


FIG. 3.—Effect of the repeated daily injection of 100 mg. S37 (as homogenate) on the liver catalase activity of (*a*) normal females, and (*b*) adrenalectomised females. The control groups (no S37) appear on the left-hand sides of the graphs (abscissa points 0, 0). In the adrenalectomised females the second dose was reduced to 70 mg.

of catalase activity is much lower, the same ultimate point is reached, i.e., some 60 to 65 arbitrary units/mg. N. From the third graph in Fig. 2 it may be seen that this is, in fact, the level reached after castration and adrenalectomy, and that the injection of tumour homogenate into castrated and adrenalectomised animals had little or no effect on catalase level. This was despite the fact that the mortality rate amongst castrated and adrenalectomised mice injected with tumour homogenate was extremely high, the survivors making up only 2 full groups. These results suggest most strongly that the extent to which catalase activity can be depressed by tumour homogenate is sharply limited, and it is that part of the total activity governed by the testicular-adrenal hormones which is affected.

Normal females treated with 100 mg./day doses of tumour are similarly affected (Fig. 3). The catalase level falls, but only down to about 60 arbitrary units/mg. N. This is only slightly below the level reached after adrenalectomy.

The similarity between the response of normal females and castrated males is well illustrated in Fig. 2 and 3.

Fig. 3 also shows that the effect of injected tumour homogenate on adrenalectomised females is small. However, the toxicity of the tumour tissue in these mice was even higher than in the males, 20/35 mice dying within 24 hours after the first injection of 100 mg. In an attempt to conserve the survivors, the second dose was reduced to 70 mg., and this was successful, there being no further deaths.

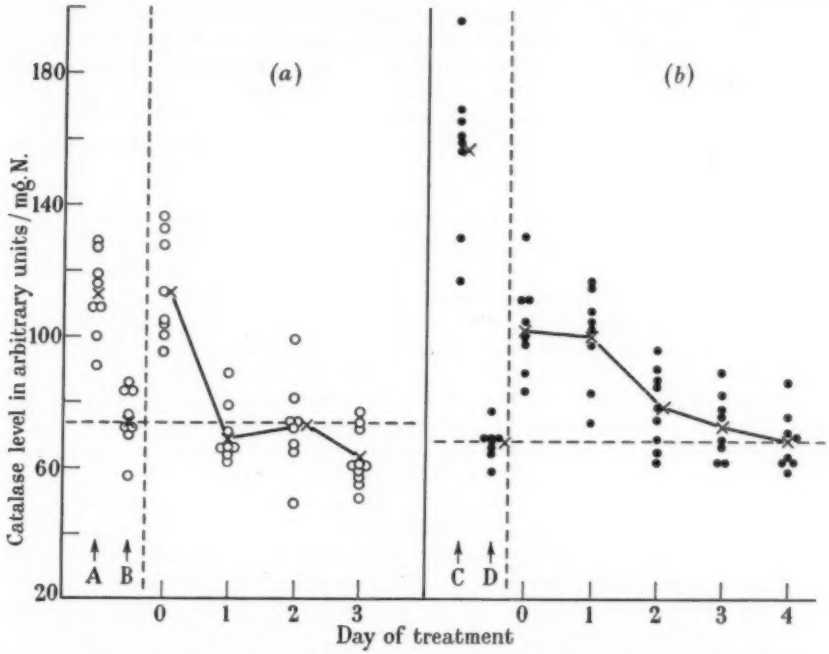


FIG. 4.—Effect of the repeated daily injection of 100 mg. S37 (as homogenate) on the liver catalase activity of (*a*) adrenalectomised females, (*b*) castrated and adrenalectomised males; both groups were treated with 50 μg. of cortisone daily beginning 5 days before tumour injection. The control groups (no S37) appear at abscissa points 0, 0. The catalase levels of normal and operated animals are shown for comparison at the extreme left of each graph. (A, normal females, B, adrenalectomised females; C, normal males; D, castrated and adrenalectomised males).

In order to obtain some direct evidence bearing upon the inhibition of cortisone stimulation by tumour injection a group of female mice was adrenalectomised, and the normal catalase level restored by the daily injection of 50 μg. of cortisone for 5 days. Sarcoma 37 was then injected daily in 100 mg. doses (the cortisone treatment being continued). As Fig. 4 shows, there was an immediate sharp fall in catalase activity to around 65 arbitrary units, the level remaining constant at this figure. There was some mortality following the injection of tumour, but the rate (6/35 in 24 hours; no subsequent deaths) was considerably lower than amongst the adrenalectomised mice not treated with cortisone.

Although it was shown (Adams, 1952) that cortisone and testosterone exert independent effects on catalase, evidence was obtained favouring the supposition that there is some adrenal mediation in testosterone secretion. In view of this possible interaction between the testicular and adrenal systems, it was thought better to use castrated and adrenalectomised animals treated with cortisone for the examination of the tumour-cortisone relationship in males. The results appear in Fig. 4. As previously found (Adams, 1952), cortisone treatment of these animals did not restore the catalase activity to normal, although an appreciable rise was observed. As in the adrenalectomised, cortisone-treated females a fall in catalase activity resulted from the daily injection of tumour homogenate. Although the fall in activity after tumour injection occurred more slowly than in the adrenalectomised female mice treated with cortisone, the extent of the fall was again approximately equal to the rise induced by the cortisone treatment. In this group of mice there were no deaths for 48 hours following tumour injection.

## DISCUSSION.

The results confirm the previous observation (Adams, 1950*a*, 1951) that the immediate depression in liver catalase activity following the injection of tumour homogenates is greater in males than in females. Castrated males behave similarly to normal females after tumour injection. Castrated males and normal females react as normal males after their catalase activity has been raised by testosterone injection. The continued injection of tumour tissue into normal males lowers their catalase activity approximately to the point reached after castration and adrenalectomy, i.e., 60 to 65 arbitrary units/mg. N. Castrated animals treated in the same way reach the same ultimate catalase level despite their lower initial activity. Adrenalectomy of castrated animals reduces the liver catalase activity to around 65 units as stated above, and the subsequent injection of tumour produces little or no change. In adrenalectomised females the final level and behaviour towards tumour injection is similar to that of castrated and adrenalectomised males. Restoration of the adrenal-controlled component of the catalase activity by cortisone injection restores the sensitivity to tumour. In each case the removal, or absence, of the gland results in a loss of sensitivity to the catalase-depressing effect of tumour injection, which is quantitatively restored by the injection of the appropriate hormone. It is possible, however, that there is some other small component of the catalase activity which is tumour-sensitive—some of the levels in tumour-treated animals were slightly lower than those after adrenalectomy in females or castration and adrenalectomy in males. Possibly there is some direct pituitary contribution. Nevertheless, it seems that by far the greater part of the liver catalase activity which is sensitive to tumour homogenates is normally under adrenal and testicular control. The effect of a large growing tumour is a little greater than that of tumour homogenates, since average catalase levels of about 45 arbitrary units have previously been recorded in S37 bearing mice (Adams, 1950*a*). The gap may well be due to non-specific changes in nutritional status associated with tumour growth.

The important feature of the present results lies in the blocking effect of the catalase-depressing substance on hormonal stimulation of the liver, particularly since an essential substance such as cortisone is involved. How many of the functions of cortisone in the liver suffer interference is not known, but it would

seem unlikely that the effect is confined to liver catalase. The concentrations of other liver enzymes and components are known to be altered in the tumour-bearing animal (Greenstein, 1947). It seems reasonable to suppose that at least some of these changes may be effected in the same way as the depression of liver catalase—i.e., through an interference with systemic influences. However, the main interest of these liver changes is in the possibility that they may throw light on processes occurring within tumours, particularly on those responsible for their "autonomous" nature. Greenstein (1947) has pointed out that the changes which occur in the liver of a tumour-bearing animal are in the direction which the liver would take if it were transformed into a hepatoma. The isolation of the liver from systemic controlling influences may be interpreted as a step towards autonomy. Possibly, therefore, the function of the catalase-depressing substance within the tumour is to protect it against interference from systemic influences, and this substance may be responsible for the autonomous nature of tumour growth.

It is not yet known whether cortisone and testosterone are capable of exerting any protection against the catalase-depressing substance of tumours. According to Begg (1951) testosterone does not restore the catalase level in rats bearing large tumours, but non-specific nutritive effects may interfere under these conditions.

Fukuoka and Nakahara (1951) have recently reported the interesting observation that liver catalase is restored to normal in tumour-bearing mice by feeding or injecting ferric chloride. These authors suggest that tumours may interfere with the incorporation of iron into liver catalase, and that other iron-containing enzymes may be found to be affected. It would be interesting to investigate the relationship between iron and the hormones in the maintenance of liver catalase activity.

SUMMARY.

The liver catalase activity of normal male mice, which is higher than that of female or castrated male mice, is depressed to a greater extent than that of the latter by injection of homogenised S37 tissue.

Normal females and castrated males treated with testosterone (which raises their liver catalase level) react to tumour injection like normal males.

There is a limit to the capacity of injected tumour tissue to depress liver catalase activity, the final enzyme level being approximately that reached after castration and adrenalectomy in males, or adrenalectomy in females.

Injection of tumour into adrenalectomised females, or castrated and adrenalectomised males, results in little or no further depression in catalase activity.

Injection of cortisone into adrenalectomised females, or castrated and adrenalectomised males, raises the liver catalase level. Injection of tumour into animals treated in this way depresses the level again, and the extent of the fall is approximately equal to the rise produced by cortisone.

The substance produced by tumours which depresses liver catalase activity appears to act by protecting this liver enzyme from hormonal influences. It is suggested that other changes in the liver of tumour-bearing animals may be produced in a similar way, and the hypothesis is advanced that the substance has a protective function within the tumour itself.

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# TRANSPLANTATION OF SKIN COMPONENTS DURING CHEMICAL CARCINOGENESIS WITH 20-METHYLCHOLANTHRENE.

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IT is perhaps natural that in the study of experimental epidermal carcinogenesis attention should have been concentrated largely on the epidermis itself, and many workers, e.g., Pullinger (1940), Glucksmann (1945), Salaman and Gwynn (1951), have endeavoured to find evidence of specific changes in the epidermis during the so-called induction period. Others, including Kreyberg (1929), Handley (1931), Orr (1937, 1938) and Howes (1946), have drawn attention to changes which are to be found in the deeper tissues. The recent development of skin-grafting techniques in laboratory animals by Medawar and his colleagues (Billingham and Medawar, 1951) has led us to investigate the possibilities of determining the relative importance in carcinogenesis of the epithelium and deeper tissues by the transference of superficial layers of skin between body-sites treated with methylcholanthrene and untreated. It was hoped in this way to obtain information as to how far the inception of malignant change in the epidermis is caused by the direct action of the carcinogen on the cells, and how far it is a consequence of changes brought about in their environment.

In a previous communication (Billingham, Orr and Woodhouse, 1950) the early results of experiments on the transference of skin from carcinogen-treated sites to untreated sites were given. It was found that transference of the treated epidermis alone did not result in the appearance of tumours at the recipient site, whereas when the treated site was regrafted with untreated epidermis tumours resulted in the ordinary way. The present communication gives further details of this experiment, and also gives an account of the results of further skin transplantation experiments which have been carried out.

*The anatomy of mouse skin.*

The integument of the greater part of the mouse's body is very mobile. It is composed of a very thin and delicate superficial epidermis which rarely exceeds more than about two layers of more or less isodiametric Malpighian cells in thickness with only a very thin cuticular layer above, the entire thickness rarely exceeding 20$\mu$. Its appendages, the hairs and their sebaceous glands, lie in the dermis or corium, consisting mainly of stout collagen fibres in a three-dimensional packing. The " panniculus adiposus", or subcutis, a layer of fatty tissue, which is equivalent to the superficial fascia in other mammals and varies greatly in thickness according to the phase of the hair-growth cycle, is firmly united to the

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dermis above and to the " panniculus carnosus", a layer of striped muscle, below. This muscle layer is very loosely attached to the underlying body wall by areolar connective tissue.

The principal vessels and nerves of the skin run in a plane parallel to its surface at the junction between the panniculus adiposus and panniculus carnosus. Though the sebaceous glands are housed in the superficial layers of the dermis, the actual bases of the hair-follicles penetrate very much deeper and may reach, or occasionally penetrate, the panniculus carnosus.

In the relatively hairless skin of the mouse's tail the epidermis is very much thicker, 60$\mu$ or more, and shows a clearly defined stratification. A very thick and compact stratum corneum is present.

*Operative methods.*

For convenience we give a general account of operative methods, and refer to modifications and special techniques in the sections describing individual experiments.

*Anaesthesia.*—All operations were carried out under sodium pentobarbitone anaesthesia, supplemented where necessary with ether. Nembutal (Abbott Laboratories) was diluted 1 in 10 with normal saline. Of this diluted solution 0·1 ml. per 10 g. of body weight was injected intraperitoneally.

*Pre-operative preparation of skin.*—The treatment of both donor and recipient areas was exactly the same. The hair was clipped short, and the skin shaved clean with a Durham Duplex razor. After removal of the soap with surgical spirit the skin surface was swabbed with a 0·1 per cent solution of " Cetavlon " (cetyltrimethylammonium bromide) in 70 per cent alcohol which was allowed to dry on. The various operative procedures to be described were carried out aseptically.

*The structure and preparation of the grafts.*—Skin *autografts* were used throughout this study, i.e., skin grafts which were transplanted back to the same animal from which they were cut. Two main types of graft have been used, the pinch graft and the Thiersch graft.

The pinch graft is a disc of skin, 10 to 12 mm. in diameter, and includes the entire thickness of the dermis. The skin of the prepared donor area, which in our experiments was that of the dorso-lateral thoracic wall, was raised into a cone or " tent " with the aid of fine watchmaker's forceps, the points of which had been so adjusted that they could be approximated in a pincer-like action. This tent of skin was then removed by slicing through its base with a No. 12 curved scalpel. The diameter of the resultant graft depended on the height to which the tent of skin was raised before cutting. In the mouse, unlike the rabbit, the skin is very firmly adherent to the panniculus, so that pinch grafts almost invariably include more or less of the underlying adipose and muscle layers. These layers were carefully snipped off with fine curved scissors to facilitate the sound healing of the graft.

The Thiersch graft as used in our experiments is essentially a very thin approximately rectangular shaving of skin usually about 10 mm. $\times$ 5 mm., comprising the epidermis and only the very superficial part of the dermis. It does not include the bases of the hair follicles. The skin of the prepared donor area was held as taut as possible over the finger and a series of thin shavings was sliced

off with a No. 11 straight-edged scalpel. The cutting and subsequent handling of these grafts was facilitated if the donor area had been first lightly smeared with sterile vaseline.

In the experiments to be described two distinct thicknesses of skin shavings have been used. These will be referred to as *thin* Thiersch grafts and *thick* Thiersch grafts respectively, depending on the amounts of dermis they included. Thin Thiersch grafts were the thinnest shavings it was possible to cut, and only rarely did they include even the sebaceous glands of the hair follicles, whereas thick Thiersch grafts nearly always included these, though they did not normally contain the follicle bases.

Thiersch grafts being very much thinner than pinch grafts heal very rapidly. Pinch grafts regenerate a full pelt of hairs, and depending on their orientation in the bed they could readily be identified by the consequent disorientation of hairs, even long after transplantation. The grafts were kept raw-side down on a piece of sterile filter-paper moistened with Ringer's solution while the recipient bed was being prepared.

The margins of the donor area of a pinch graft were approximated with two or three fine silk sutures. In the case of the donor areas of the Thiersch grafts these were dusted thickly with sterile sulphadiazine powder, no special dressings being required. These areas were very rapidly resurfaced by the migration of epithelium from the transected hair follicles and the margins of the wound.

*Preparation of the recipient area.*—The grafts were transplanted to a recipient area cut in the dorso-lateral skin of the animal's chest, where the ribs afforded a firm substratum. In rodents the most favourable bed for grafts is the vascular fascial plane overlying the panniculus carnosus. Unfortunately, in the mouse, stripping the skin down to this layer is difficult owing to the firm union that exists between it and the overlying tissues. To receive Thiersch grafts an elongated bed about 10 mm. × 12 mm. was cut. A very small pinch graft was first removed from one corner of the intended area, after which the skin edge was picked up with fine forceps and carefully dissected free from the underlying vascular plane in parallel strips. A series of Thiersch grafts was then placed as close together as possible on the prepared bed so as to cover it completely.

The pinch graft was transplanted to a hole cut in the skin of the chest of such size that it was an exact fit. This was effected by placing the graft in the defect left behind after cutting from the recipient area a pinch graft slightly smaller than the one to be transplanted, to allow for the slight gaping of the wound. However carefully such a bed was prepared, at least the central portion of the vascular fascial plane was inevitably removed. A preliminary series of trials proved that this did not prejudice the healing-in of the grafts.

To facilitate recognition of the operation field after considerable intervals of time Indian ink was lightly stippled into the intact skin with a fine needle around the perimeter of the prepared bed.

*Dressings.*—Immediately after placing the graft on the prepared bed the entire operation field was dusted with sterile sulphadiazine. A rectangular sheet of fine-mesh "tulle gras" (vaseline-impregnated gauze) was placed over the grafted area. Finally, to achieve the appropriate degree of vertical pressure over the graft and prevent its lateral displacement a 7-in. length of "Gypsona" plaster-impregnated $\frac{5}{8}$-in. bandage was wound firmly round the entire thorax. This adhered to the hairs and formed a firm jacket holding the graft in place.

The animal's attempts to gnaw its dressings were discouraged by painting with picric acid solution.

*Primary inspection* was carried out after 10 to 12 days. By this time the process of healing-in was complete, and provided that the graft had been a satisfactory fit, it was found to have established a clear suture-line with the skin surrounding it. The increased vascularity of the newly healed-in graft was now beginning to subside, though the epidermis remained hyperplastic. At this stage a thick cuticular layer of keratinized epithelial cells could usually be stripped away from the graft surface—the so-called "ghost" graft (Billingham and Medawar, 1951). This included the original hairs of the graft, which had been shed from the now cystically dilated follicles.

After the primary inspection the young graft was protected by plain bandage secured in position with a length of plaster bandage. This temporary dressing was removed after about 10 days, no further dressings being required. In the case of the pinch graft new hairs had usually begun to pierce the graft surface by about the 20th day, by which time the epidermis had reverted to its original thickness. A normal pelt of hairs was present on the grafts 30 to 40 days after transplantation.

*Preliminary carcinogenic treatment.*

White mice of mixed stock were used. They were kept in metal boxes, 4 animals to a box, and were fed on the ordinary laboratory diet of rat cubes obtained from Heygate & Sons (known as the Thompson diet). They were painted with 0·3 per cent solution in acetone of 20-methylcholanthrene once a week. The solution was kept in the dark to avoid photo-oxidation. Paintings were continued for 12 weeks. The applications were made with a glass pipette. The first application was carefully made on a site on the right side of the scapular region, well away from the dorsal mid-line and using 0·02 ml. of solution. Subsequent applications to the centre of the resultant epilated area (about 1·5 cm. diameter) were of approximately 0·04 ml. each. Care was taken that spread of the solution beyond the original area did not occur. After the full course of this treatment the animals were left for two weeks before operation. There are good grounds for believing that in this space of time methylcholanthrene would have completely disappeared from the body of the animal, and that when the grafts were made there was no likelihood that methylcholanthrene was transferred at the same time as the graft, nor that it was still present in the deep tissues of the treated site.

Nodules which appeared and subsequently regressed are not counted as tumours. This phenonemon does not occur frequently, but it does not seem wise to regard as neoplastic a nodule which lacks the property of persistent growth. In at least one case a nodule on the grafted area has been shown to be an inclusion dermoid.

Tumours which arise below the treated area have been seen. This is evidence of the impossibility of localizing the treated area with exactitude, and would account for an occasional heterotopic tumour.

Difficulties may arise as a result of shrinkage of scar tissue in determining whether a tumour which is in a different place at successive examinations is in fact the same tumour. Similar difficulties may arise from the way in which the mouse is held, owing to the mobility of the skin.

It was regarded as important to remove tumours from the treated area in certain experiments in order to prolong the life of the mouse and give the grafted area the fullest possible chance of developing a tumour.

*Experiment A : Transplantation of Untreated Tail Skin Epidermis to Recipient Area Cut in Carcinogen-treated Skin.*

By the tryptic dissolution of the fine elastic fibres that unite the epidermis to the dermis morphologically intact sheets of " pure " epidermis can be prepared in which the cells are alive, as evidenced by the fact that sheets of epidermis so prepared can be successfully transplanted (Medawar, 1941 ; Billingham and Medawar, 1951).

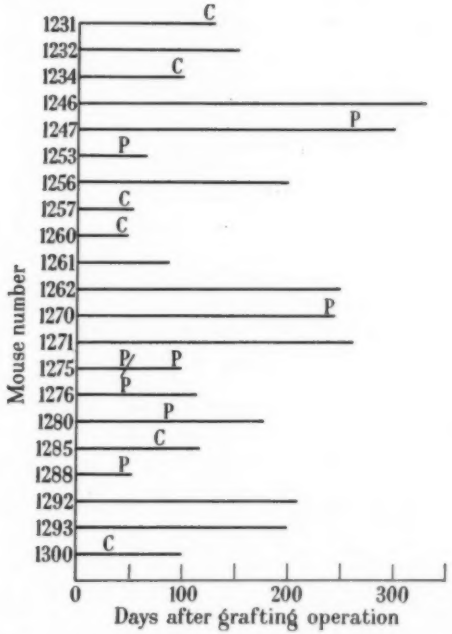


FIG. 1.—Rate of appearance of tumours (Experiment A) in methylcholanthrene-treated skin after removal of original epithelium and grafting of untreated tail-skin epidermis.
P = papilloma.
C = carcinoma.
Each horizontal line indicates the time of survival of an animal after grafting. The oblique line indicates operative removal of the tumour preceding it.

The object of this series of experiments was to remove the epidermis from the carcinogen-treated area on the right side of the animal's thorax as completely as possible, and then to replace it with sheets of pure epidermis prepared from normal untreated skin elsewhere on the body. Sheets of epidermis prepared from the skin of the tail were selected, since they are thicker and easier to handle than

those prepared from the general integument, where the abundant hair follicles interfere with the clean fission of the dermis from the epidermis.

Several thin rectangular Thiersch shavings of even thickness, about 5 mm. × 6 mm., from the skin of the tail were floated on trypsin solution (a Seitz-filtered 0·5 per cent solution of commercial trypsin powder in Ringer-bicarbonate adjusted to pH 7·8) and incubated at 38° C. for 30 minutes. Longer periods were required for thicker shavings. After rinsing in Ringer's solution the skin shavings were carefully spread out, cuticular side down, in a dry sterile Petri dish. The dermis could then be peeled off very easily with fine forceps, leaving the epidermis as an intact sheet.

The epidermis from the carcinogen-treated skin on the animal's thorax was removed simply by cutting a series of very thin Thiersch shavings from it. This operation was greatly facilitated by the rather oedematous thickening due to the carcinogen treatment. A drop of Ringer's solution was placed on the denuded area and on this the pure epidermal grafts were floated. After withdrawal of the excess fluid with a piece of sterile filter-paper the grafts were adjusted in position so as to cover the prepared area as completely as possible. After dusting the operation field with sterile sulphadiazine powder standard dressings were applied.

At the primary and subsequent early inspections satisfactory evidence was obtained that the grafts had taken. By about the 10th day thin cuticular "ghosts" could be peeled away from the grafts to reveal whitish healthy sheets of epithelium, which by their proliferative outgrowth had frequently coalesced.

Since in the preparation of the graft bed the greater portion of the thickness of the dermis was left intact, the bases of the follicles remained behind and so the epithelium of the grafted area was unavoidably of dual origin. Some hairs eventually pierced the surface. It was not possible to distinguish histologically between the transplanted epithelium of tail skin origin, and that originating from truncated follicles left behind in the graft bed.

The results of subsequent periodical inspection of the mice are shown in Fig. 1. One animal did not survive operation up to the time of first tumour appearance, and has not been included. Of the remaining 21 mice, it will be seen that 13 animals developed tumours on the treated area in from 35 to 265 days after grafting, with an average time of 92 $\pm$ 18·5 days. In 2 cases (1270 and 1276) the tumour showed sebaceous differentiation, in one (1234) it was anaplastic. In the remaining 10 animals the tumour was squamous papilloma or carcinoma with keratinization, and appeared on histological evidence to have arisen from the superficial epidermis itself (Fig. 2 and 3). Some of the tumours were found at skin sites where no hair follicles were present.

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EXPLANATION OF PLATES.

FIG. 2.—Squamous carcinoma (Mouse 1260) arising from the superficial epidermis. × 35.

FIG. 3.—Squamous papilloma (Mouse 1253) arising from superficial epidermis. × 40.

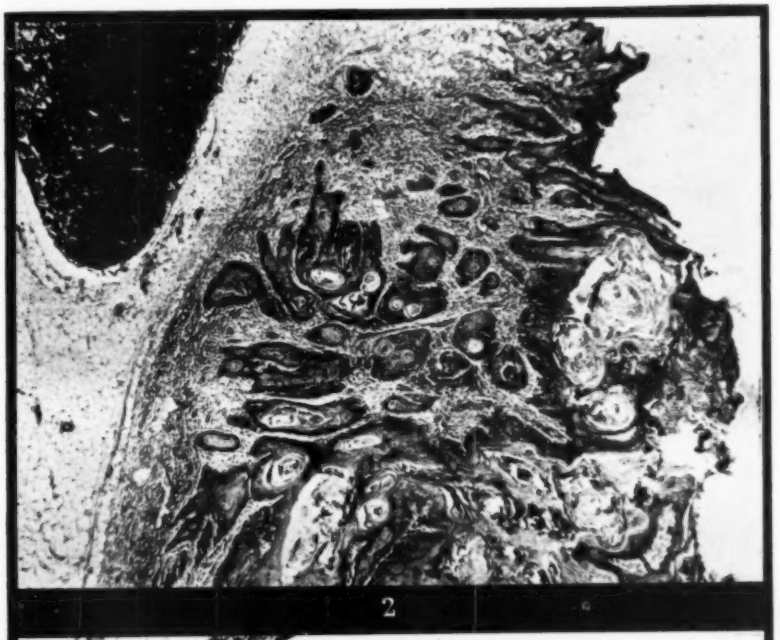
FIG. 6.—Tumour with sebaceous differentiation (Mouse 1279). × 80.

FIG. 7.—Tumour possibly arising from hair follicles (Mouse 1284). × 82.

FIG. 9.—Epidermal papilloma from grafted area (Mouse 1393). Elastic tissue stained. Note altered character of dermis below tumour base. × 45.

FIG. 10.—Epidermal carcinoma from grafted area (Mouse 1346). Elastic tissue stained. Beneath the tumour the dermis has been completely replaced by grafted dermis containing practically no elastic tissue. × 24.

FIG. 11.—Mouse from Experiment G, to show disorientation of hair growing from graft in left dorso-lateral thorax.



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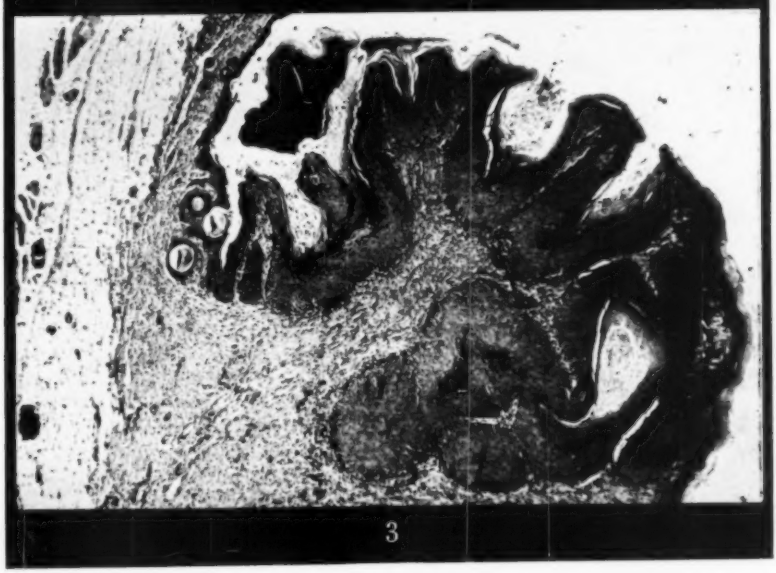

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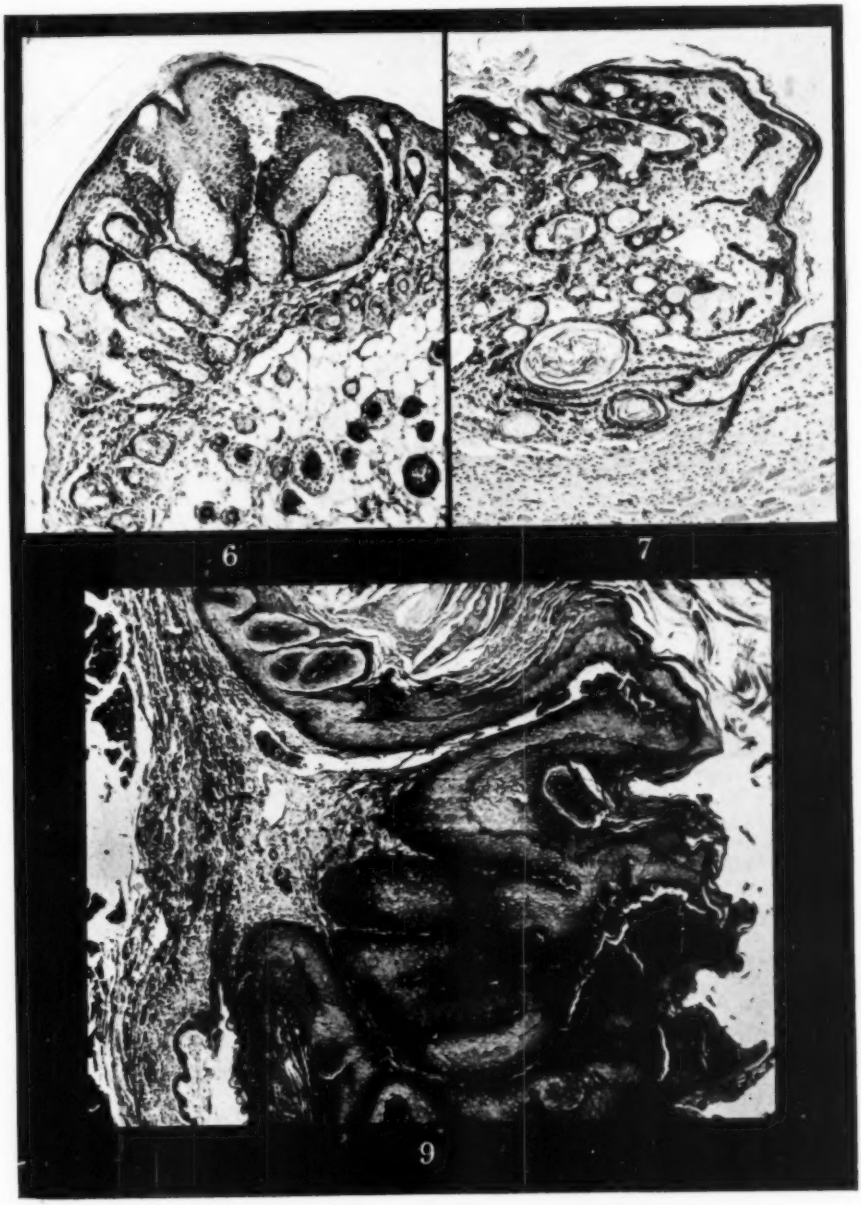

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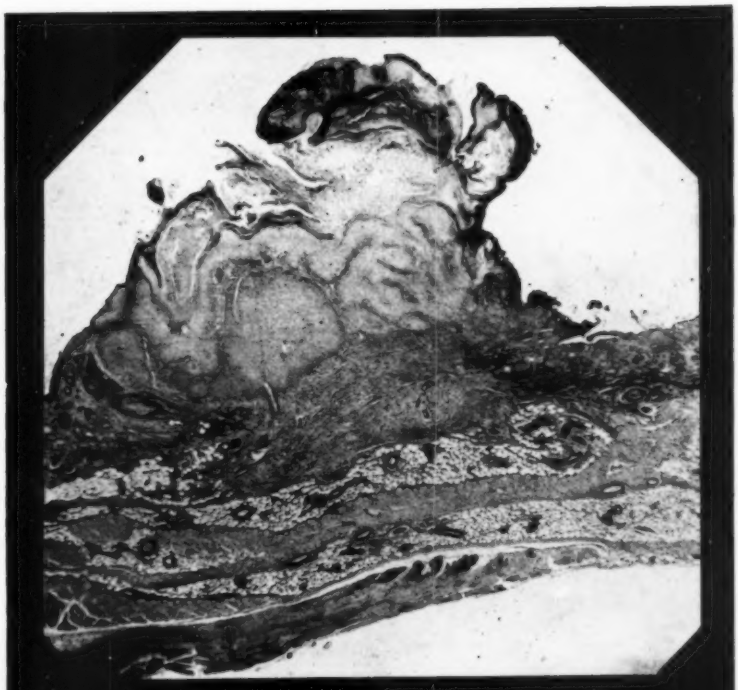

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Billingham, Orr and Woodhouse.

*Experiment B : Transplantation of Carcinogen-treated Epidermis to a Recipient Area Cut in Normal Skin.*

The object of this series of experiments was to investigate whether sheets of pure epidermis prepared from the carcinogen-treated skin on the right side of the animal's chest would give rise to tumours when transplanted to a bed cut in normal untreated skin—in our experiments, in the skin on the opposite side of the chest. The sheets of pure epidermis were prepared exactly as in Experiment A

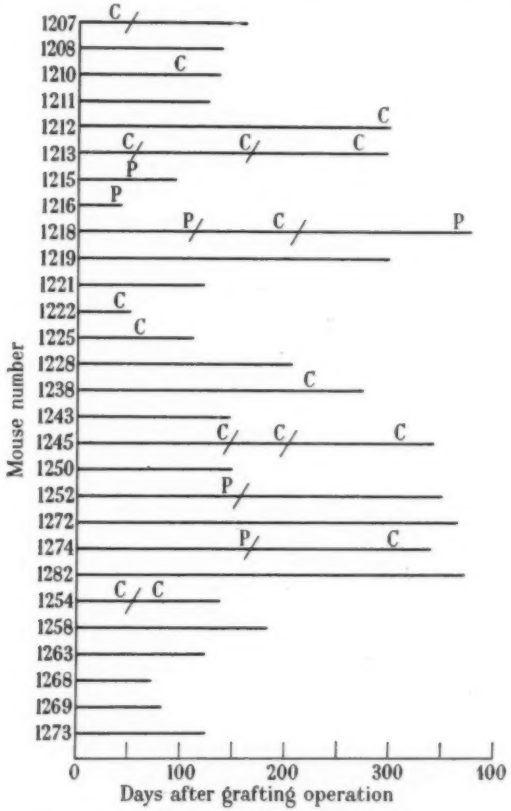


FIG. 4.—Rate of appearance of tumours (Experiment B) in original methylcholanthrene-treated area after removal of thin Thiersch grafts for transplantation elsewhere. Notation and symbols as in Fig. 1.

from thin shavings of skin cut from the treated area. The changes evoked in this skin by the application of the carcinogen greatly facilitated both the cutting of the thin shavings and the subsequent tryptic separation of the dermis from the greatly thickened epidermis.

The resultant pure epidermal grafts were transplanted to a " half-thickness " bed cut in the normal skin on the left side of the animal's chest, by the removal

of a series of very thin superficial shavings. As in the previous series of experiments the epithelium resurfacing such a grafted area is unavoidably of dual origin.

It should be added that pure epidermal grafts do unite firmly, and in a functionally adequate way after transplantation to either a freshly cut full-thickness bed, from which all dermal tissue has been removed, or to a similar bed which is already granulating. Unfortunately such an operation field, despite the fact that it has been completely resurfaced by the transplanted epithelium, undergoes progressive contracture, which ultimately results in the approximation of the original margins of the wound. The fate of the transplanted epithelium is at present obscure (Billingham and Medawar, 1951). If, however, sufficient dermal collagen is left behind when the bed is prepared, as was done in our experiments, contracture of the wound does not take place and the pure epidermal grafts manifestly survive.

The results are shown in Fig. 4. Four animals did not survive operation up to the time of first tumour appearance, and have not been included. *No tumours arose on the grafted site in the remaining 28 mice.* Tumours appeared on the original treated site in 14 animals. In some cases these tumours were removed to prolong the life of the mouse, and in some of these further tumours occurred, but never on the grafted site. The time range from operation for the appearance of a first tumour was from 37 to 272 days after operation, with an average time of 106 $\pm$ 20 days. Of the 22 tumours in all (including those subsequent to the first in each animal), 3 (1215, 1252, and the third tumour in 1218) showed sebaceous differentiation, in 2 (1207 and the third tumour in 1245) origin from the hair follicles could not be excluded, and one was anaplastic. The remaining 16 tumours appeared histologically to be squamous and horny papillomata and carcinomata of superficial epidermal origin. The survival of the mice after grafting ranged from 41 to 376 days, with an average time of 197 $\pm$ 20·4 days ; one would therefore infer that an adequate opportunity had been given to the grafted epithelium on the left side of the thorax to reveal its neoplastic potency if present.

*Experiment C : Transplantation of Thin Thiersch Grafts of Carcinogen-treated Skin to a Recipient Area Cut in Normal Skin.*

In these experiments 4 thin Thiersch grafts were cut from the treated skin on the right side of the animal's chest and transplanted to a full thickness bed cut down to the vascular fascial plane in the untreated skin of the opposite side of the chest, the aggregate area of skin so transplanted being 1 to 2 sq. cm., depending on the size of the animal. Whereas in most animals the grafts were transplanted to a common bed, in a few each of the 4 grafts was transplanted to a separate full-thickness bed, the individual beds being separated by small stretches of intact skin. As some of these grafts eventually bore hairs, it seems possible that the operative aim was not fully achieved, and that either the bed or the graft must have contained hair bulbs.

The results are shown in Fig. 5. Three animals (two single-bed and one multiple-bed graft) did not survive operation up to the time of first tumour appearance, and have not been included. In the remaining 15 mice no tumours appeared on the grafted sites, although in one instance (1284) the tumour arose close to the boundary between the two sites and was accurately localised only

after histological examination. There was one other lesion on a grafted site (1223) which proved on histological examination to be an implantation epidermoid cyst. Tumours appeared on the original treated area in 7 animals, and some were removed in order to prolong the survival time of the mouse. The mean time after operation of tumour appearance was 38 ± 7·5 days. Of the 9 tumours in all, 2 (1223 and the second tumour of 1279) showed some sebaceous differentiation (Fig. 6) and in one (1284) an origin from hair follicles could not be excluded histologically (Fig. 7); the remaining 6 showed origin from the superficial epidermis, and 2 of these were arising in a part of the skin from which hair follicles were absent.

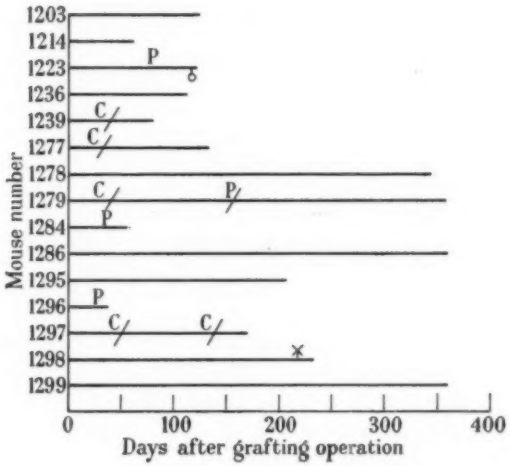


FIG. 5.—Rate of appearance of tumours (Experiment C) in original methylcholanthrene-treated area after removal of thin Thiersch grafts.

× (in Mouse 1298) = mammary carcinoma.
○ (in Mouse 1223) = implantation epidermoid cyst in grafted area.

Remaining notation as in Fig. 1.

### *Experiment D: The Transplantation of Thick Thiersch Grafts of Carcinogen-treated Skin to a Recipient Area Cut in Normal Skin.*

Apart from the fact that the grafts were thicker than those used in the experiments described in (C) above no further comment is necessary. In every case the grafts were transplanted to a common full-thickness bed.

The results are shown in Fig. 8. One animal did not survive operation up to the time of first tumour appearance, and has not been included. In the remaining 30 mice tumours appeared on the grafted site in 5 animals, and on the original treated site in 5 animals. Two animals bore a tumour at each site. The time after operation of appearance of tumours at the grafted site ranged from 21 to 90 days, with a mean time of 53 ± 11·5 days. The corresponding values for tumours on carcinogen-treated sites are 21 to 392 days (mean 186 ± 75). In

3 of the tumours on grafted sites there was histological evidence that the entire thickness of the recipient dermis had been removed in the region where the tumour arose, and replaced by more or less the entire thickness of the transplanted dermis (Fig. 9 and 10). This is regarded as a point of some importance in finding

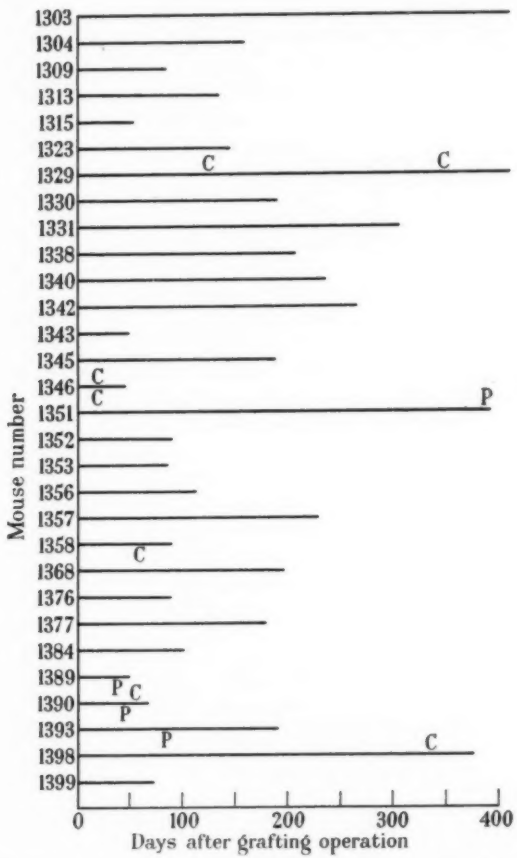


FIG. 8.—Rates of appearance of tumours (Experiment D) in methylcholanthrene-treated donor area and untreated recipient area after transference of thick Thiersch grafts.

Letters above the life-line = tumours on original treated area.
Letters below line = tumours on grafted area.

Notation otherwise as in Fig. 1.

an explanation for the discrepancy between this experiment and Experiment C, and the fact that the grafts were cut thicker may also account for the lower yield of tumours on the original treated site. None of the tumours showed evidence of origin from hair follicles or sebaceous glands.

*Experiment E : The Exchange of Full-thickness Grafts between the Treated and Untreated Sides of the Chest.*

Standard " pinch " grafts were cut from the normal skin and the treated skin on the left and right sides respectively of the animal's chest. After trimming off all remnants of the muscle and fatty layers each graft was transplanted to the bed on the opposite side of the body, so that the graft of carcinogen-treated skin was transplanted to a defect in normal skin and *vice versa*. Under these conditions the grafts were unavoidably rather " open " fits, an annulus of exposed raw tissue lying between the rim of the graft and the margin of its bed. The healing of these grafts was perfectly satisfactory, re-surfacing of the annulus of raw tissue being brought about by the outward migration of epithelium from the graft and the inward migration of epithelium from the margin of its bed. A slight modification of the standard dressings was necessary in these bilaterally operated animals. Instead of applying separate rectangles of tulle gras over the two operation fields, a 3-inch strip of rather coarser mesh tulle gras (prepared by impregnating $\frac{5}{8}$-inch open-wove bandage with vaseline) was wound round the entire thorax so that it covered both areas, after which a length of plaster was applied.

Of the 14 mice which survived operation up to the time of first tumour appearance, 3 developed tumours (in 55, 55 and 85 days after operation), all on the original treated site. In 2 of these removal of the tumour was followed by the appearance of a further tumour. No tumours appeared on the site which had received carcinogen-treated grafts. Six of the mice were still alive 211 days after operation. Only one of the tumours showed histological evidence of origin from hair follicles and sebaceous glands ; 4 of the 5 were malignant. Histological examination of both sites in a mouse which died 12 days after operation showed that while the graft of treated skin to the untreated site was apparently fully viable and had " taken", the reciprocal graft of untreated skin to the treated site was necrotic throughout its thickness.

*Experiment F : Reflection of a Full-thickness Flap of Skin from the Treated Area, followed by Resuturing into its Original Position.*

In the experiments so far described, as in those of the following sections, " free " skin grafts have been used, i.e., grafts which have been completely freed from all connections with the body. Such grafts have perforce to suffer a period of ischaemia before the re-establishment of a vascular supply after transplantation. The object of this series of experiments was to effect the partial but not complete interruption of the vascular system of the graft. This was effected by making incisions through the skin down to the body wall along three sides of a rectangle, about 1·5 cm. cephalo-caudally × 2 cm. dorso-ventrally, which included practically the entire treated area. The fourth side of the rectangle, which ran along the dorsal midline, was not incised, being left as a pedicle on which the graft (in this case including the panniculus carnosus) was reflected from the body wall. After this it was replaced and sutured in its original position with interrupted fine silk sutures. Standard dressings were then applied. The healing of these flap grafts was uneventful.

Of 6 mice which survived operation up to the time of first tumour appearance, 2 developed tumours (in 53 and 57 days after operation). At the time of writing 2 mice are alive 161 to 168 days after operation, without tumours. Both tumours

appeared to arise from the superficial epidermis ; one was a papilloma, the other a carcinoma.

*Experiment G : Transplantation of a Full-thickness Graft of Treated Skin to a Recipient Area Cut in Normal Skin.*

This series is in effect a repetition of series (E), except that no attempt was made to transplant a graft of normal skin to the treated area. Every attempt was made to cut as large a graft as possible from the treated area, and to ensure that it fitted exactly into the bed cut to receive it in the normal skin on the left side of the animal's chest. These grafts were readily detected for many months by the disorientation of hairs (Fig. 11).

Of 23 mice which survived operation up to the time of first tumour appearance, 3 developed tumours (in 44, 102 and 162 days) on the original treated area, and 3 (in 46, 84 and 98 days) on the grafted area. One animal, included in both these groups, bore tumours on both sites. At the time of writing 17 mice are still alive, having survived operation for periods of 126 to 168 days.

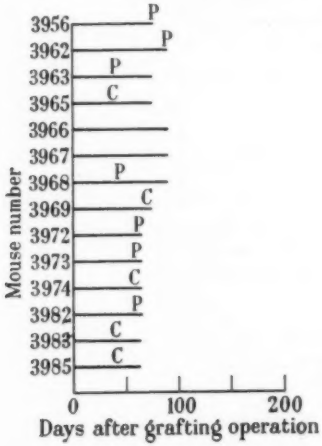


FIG. 12.—Rate of appearance of tumours (Experiment H) in methylcholanthrene-treated skin after detachment and re-implantation. Notation as in Fig. 1.

*Experiment H : The Re-implantation of a Pinch Graft Cut from the Carcinogen-treated Skin Back into its Own Donor Site.*

In these experiments a large pinch graft was cut from the carcinogen-treated skin, all remnants of the muscle and fatty layers trimmed off, and the graft re-implanted into the defect from which it had been taken. Owing to the natural gape of the wound after cutting and the slight contraction of the graft it was a rather " open " fit. Nevertheless these grafts healed-in satisfactorily.

The effect of this procedure was greatly to augment the carcinogenic process in the group of mice tested. The results are shown in Fig. 12. Of 14 mice which survived operation up to the time of first tumour appearance, 12 developed tumours in 39 to 90 days (mean 57 $\pm$ 5 days). At 90 days the experiment was

terminated by killing the remaining mice. In addition to their high incidence and early onset the tumours in individual animals were strikingly larger than in the other experiments, and in 5 instances rapidly spread or coalesced to involve practically the whole of the treated and grafted area. Histologically 2 of the tumours (3956 and 3983) might have originated in hair follicles, and one (3963) showed sebaceous differentiation; the remaining 9 were apparently derived from the superficial epidermis. Seven were papillomata, and 5 carcinomata. In a further group of animals in which the treated area was grafted with normal skin from the left thorax, tail, or ear, the grafts took satisfactorily, but no similar acceleration of tumour formation was observed. This latter group has not yet been under observation long enough, however, to give the final results.

## DISCUSSION.

The present results offer some support to the view that the effective carcinogenic action of methylcholanthrene is not limited to the epithelium itself, but that the changes in the deeper tissues are of great importance. The most striking results obtained seem to be that no tumours arose on the grafted areas of Experiments B and C. If this finding is to be taken at its face value it implies that the treated epidermis no longer retained potential neoplastic properties after its transference to the untreated site. It is, therefore, crucial to attempt to decide whether these transplants were in point of fact effective. Clinical examination suggested that the grafts had taken adequately. To confirm this impression, small sheets of carcinogen-treated epidermis were transplanted to the centre of a large full-thickness bed. Under these conditions it was possible to observe directly the outgrowth of epithelium from the margins of the graft over the granulating surface. This is regarded as unequivocal proof of the viability of transplanted carcinogen-treated epithelium. It is not unreasonable, therefore, to believe that it survived in the conditions of Experiments B and C.

If the results obtained in Experiment A are subjected to the simplest explanation, the conclusion would be that it is not necessary for the epithelium itself to be directly acted upon by methylcholanthrene at all. We are fully alive to the fact that such a direct interpretation of the latter result is not fully justified at present, inasmuch as the deeper parts of the hair follicles were left *in situ* when the graft beds were prepared. It is now accepted that regeneration of epidermis can occur by outgrowth from the lining of hair follicles, and that new hair follicles and sebaceous glands can be differentiated from the superficial epidermis. The presence of hair follicle or sebaceous gland structure in tumours cannot therefore be used to provide conclusive proof that a tumour has originated from these sources, and similarly their absence cannot be invoked decisively to prove that tumours have originated from other sources. With appropriate reservations, however, we feel entitled to point out that the majority of the tumours in Experiment A showed no evidence of structure other than that of simple epidermis, and we are satisfied that the grafts of tail skin epidermis did in fact " take", and that there is a strong probability that it was this epithelium in which the neoplastic change took place in most cases.

In experiments where the graft included dermis as well as epidermis, the location of the tumours on grafted sites appeared to depend on the amount of dermis which had been transferred. Thus in the thick Thiersch grafts of Experiment D some tumours were obtained on the grafts, while none occurred on the

thin Thiersch grafts of Experiment C. In Experiment D the tumours on the grafted area tended to appear relatively early in the experiment, and it is possible that they may have been grafted *as tumours* (not yet visible to the naked eye). In Experiments E and G (full thickness grafts) there is little difference in the incidence of tumours as between treated and untreated sites, but the relatively low total yield of tumours suggests that in some way the carcinogenic stimulus must have been less potent in these experiments than in some of the others.

The results of Experiment H would lead one to believe that the carcinogenic potentialities inherent in methylcholanthrene-treated dermis are enhanced when it is divided and replaced *in situ* so that a healing reaction takes place. We have never seen experimental skin tumours develop so rapidly and extensively as they did in this group of mice ; in some of the animals tumours were already beginning to appear before the post-operative inspection of grafts was completed. In the light of this experiment a new explanation can be offered of the well-known results of Deelman (1927), who found that tumours appeared unusually rapidly in the track of an incision made in tarred skin, and of the similar findings of Friedewald and Rous (1950) in methylcholanthrene-treated rabbit's ears in which healing of punch holes had taken place.

None of our other experiments have given the type of result obtained in Experiment H. An important point may be that this was the only experiment in which both the implanted and recipient connective tissues had been subjected to the action of methylcholanthrene, and the former completely severed from its blood supply (Experiment F). It may be that the healing reaction between two carcinogen-damaged connective-tissue surfaces accelerated the development of decisive carcinogenic conditions.

In those experiments (B, C, D and G) in which the donor site was left to resurface itself from natural sources, i.e., without a skin graft, the new epidermis would be derived from ingrowth of the surrounding epidermis or by migration from the transected hair follicles, or from both sources. In Experiment B in particular care was taken to try to remove all the treated epidermis, so that only the hair follicles would be available as a source of epithelium which had been exposed to the action of the carcinogen. It may therefore be argued with some justification that epithelium of such derivation was the source of the tumours. This, however, would imply that epithelium of the hair follicles had been altered into a state of latent neoplasia by the action of the carcinogen, but had at the same time retained the power of re-differentiating into superficial epidermis. In our opinion this view is unnecessarily complicated, and on the principle of minimal hypothesis, attention should first be given to the possibility that epidermal carcinoma may be determined by growth and multiplication of epidermal cells, which are dependent for their nutritional requirements on a dermis and subcutis which have been altered in structure and function by a suitable agent. That there are early histologically demonstrable changes in epidermis treated with carcinogens is not denied, but it has still to be shown that they are relevant to actual tumour formation, especially in the light of the results of Experiment B.

If the essential primary carcinogenic change is in the sub-epidermal tissues, it would seem reasonable to expect that the tumour incidence would be secondarily influenced by the rate of epithelial proliferation. Considerations of this sort offer a tenable explanation of the phenomenon of so-called co-carcinogenesis (Berenblum, 1941, 1944). Croton oil, the best known co-carcinogen, produces

rapid and active hyperplasia of the epidermis, but this only exceptionally goes on to tumour production unless the skin has previously received treatment with a carcinogenic agent. The interval between the application of carcinogen and co-carcinogen can be a very long one, and in the meantime the epidermis reverts to a histological structure which is indistinguishable from normal. Clearly there must be some small proliferative activity during this period to replace the effects of attrition and loss of cells. It is suggested that the integrity of the dermis is sufficient to sustain this, but that when the rate of proliferation of epidermis is stepped up by croton oil, the deficiencies of the dermis are brought to light, and tumours begin to appear.

To the best of our knowledge grafting experiments of this type have not previously been reported in relation to experimental carcinogenesis. Silberberg, Silberberg and Hulbert (1948) have studied the effect of 20-methylcholanthrene on the transplantability of mouse skin, and have reported that treatment of skin with this agent previous to transplantation intensified the growth processes in the grafts, but unless the transplanted skin had acquired neoplastic properties their transplantability was decreased as compared with normal skin. Though the technique used involved orthotopic transplantation, their object was to study the early healing processes in the grafts which were removed at a very early stage for histological examination before they had entered the phase of tumour production. Their experiments and ours are, therefore, of limited relevance to each other.

It is premature to draw general conclusions from the work now reported, but if the failure to obtain tumours in transplanted carcinogen-treated epidermis is confirmed by future study, it must be taken into account in attempts to explain the mechanism of carcinogenesis. The crucial issues appear to be whether such grafts take, and whether the grafted epithelium survives long enough to give neoplastic change an opportunity to manifest itself. We believe that the first of these questions can be answered in the affirmative ; the second question raises implications which cannot yet be fruitfully discussed, but which would seem to be applicable also to epithelium left undisturbed in its original site of carcinogenic treatment.

SUMMARY.

A small area of skin was treated in mice with 20-methylcholanthrene in weekly applications for 12 weeks, this being a fully effective carcinogenic stimulation. Such mice, provided they had not already developed visible tumours, were used for transplantation experiments.

Carcinogen-treated epidermis, and thin Thiersch grafts of carcinogen-treated skin, did not yield tumours when transplanted orthotopically to untreated body sites.

Thick Thiersch grafts and whole thickness grafts of carcinogen-treated skin yielded a small number of tumours after transplantation.

Tumours were obtained in considerable number when a denuded carcinogen-treated area was re-surfaced with untreated epidermis.

Re-implantation of whole-thickness grafts of carcinogen-treated skin in the beds from which they were cut resulted in an enhanced rate and extent of carcinogenesis.

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# THE LOCAL GREYING OF HAIR IN MICE TREATED WITH X RAYS AND RADIOMIMETIC DRUGS

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THE exposure of living organisms to sublethal doses of radiation causes a number of changes, many of which can be also produced by radiomimetic drugs (Boyland, 1951). One of these is the local greying of the hair in mice described by Hance and Murphy (1926) and recently investigated by Chase (1949) following exposure to X rays. The same effect produced by implantation of plutonium has been described by Lisco, Finkel and Brues (1947). Chase (1949) considers that the measurement of the effect is suitable for the quantitative assay of biological effects of radiation. A very similar bleaching or greying of hair caused by injection of nitrogen mustard was described by Boyland, Clegg, Koller, Rhoden and Warwick (1948), and malignant tumours following nitrogen mustard treatment (in some cases in immediate contact with grey patches induced by the injection) have been described by Boyland and Horning (1949) and by Heston (1950).

The relationship between induction of grey hair and of cancer is investigated in the present paper. Localised greying of hair has now been induced in mice by a number of chemical agents which are known to be carcinogenic or mutagenic or both carcinogenic and mutagenic, or might be suspected of being so. The change is generally produced most readily by water-soluble substances but, with this limitation, the change might be used as a test indicating carcinogenicity. In this respect it is probably less specific, but similar to (1) the inhibition of tumour growth or body growth by carcinogenic compounds investigated by Haddow and his collaborators (Haddow, 1938), (2) the production of the specific chromosome damage described by Muller and Painter (1929) following X rays and by Darlington and Koller (1947) following mustard gas application, or (3) the increase in the mutation rate as described by Muller (1928) for X rays and by Auerbach, Robson and Carr (1947) for vesicants. The inhibition of growth and the induction of chromosome abnormalities in cells of small mammals can be observed readily with substances which are only very slightly soluble in water, but it is difficult to produce mutations in bacteria, *Neurospora* and *Drosophila* with substances which are not water-soluble.

Because the greying of hair described in the present paper is a discontinuous permanent change in a part of the soma, it had been suggested (Boyland, 1949) that it might be a somatic mutation and so analogous to the malignant change if the latter were a somatic mutation. The conception of somatic mutation in adult animals is, however, entirely hypothetical. If a mutation can only be proved by breeding and examination of the succeeding generations, then proof

of the existence of mutations in somatic cells can never be established. The term "permanent modification" might be a more appropriate description. The local greying effect, however, is almost certainly due to selective destruction of dendritic cells producing pigment, and histological examination of affected hair follicles should give more information as to the nature of the change.

## EXPERIMENTAL.

Coloured mice (of either stock or pure lines C57 and C3H) were injected intradermally at one, two or four sites on the abdomen with aqueous solutions of the substances under investigation. In the tables, A refers to Agouti stock mice and B to black stock mice. The concentrations were such that the dose was contained in 0·05 ml. of solution. The mice were examined three times weekly for changes in the skin. If the substances were injected in aqueous solution they were only considered active if greying was seen in a higher proportion of mice than following injection of water. Other mice were exposed to a narrow beam of X rays for different times so that different doses of irradiation were given to limited areas. The X-ray tube was operated at 140 K.V.P., giving rays with a half value layer of 0·2 mm. copper and 140 r. per minute.

TABLE I.—*Greying of Hair in Mice Injected with Different Concentrations of Nitrogen Mustard and Related Carcinogenic Compounds.*

| Compound. | Concentration (mg./ml.). | Strain of mouse. | Number of mice. | Sites affected (%). | Mean latent period for appearance (days). |
|---|---|---|---|---|---|
| Methyl *bis* (β-chloroethyl)-amine (HN2) in water | 0·2 | CBA | 5 | 100 | 50 |
| | 0·2 | ABC | 24 | 100 | 60 |
| | 0·02 | Stock | 4 | 50 | 54 |
| | 0·002 | ,, | 5 | 80 | 54 |
| Methyl *bis* (β-chloroethyl)-amine (HN2) in saline | 0·2 | B | 5 | 100 | 30 |
| | 0·1 | B | 5 | 100 | 25 |
| | 0·02 | B | 10 | 80 | 30 |
| | 0·002 | B | 4 | 50 | 47 |
| | 0·0002 | B | 10 | 10 | — |
| Methyl *bis* (β-chloroethyl)-amine and hyaluronidase in saline | 0·1 | B | 15 | 70 | 27 |
| β-Naphthyl *bis* (β chloroethyl)-amine (R 48) in arachis oil | 75·0 | C57 | 5 | 100 | 20 |
| *bis* (β-chloroethyl) sulphide (Mustard gas) in arachis oil | 0·4 | B | 20 | 60 | 56 |
| Mustard gas in propylene glycol | 0·4 | B | 10 | 90 | 24 |
| Mustard gas in water | 0·4 | B | 10 | 100 | 24 |
| 2:4:6-Tri (ethyleneimine) 1 : 3 : 5-triazine (R 246) in water | 0·0015 | CBA | 10 | 80 | 30 |
| 1:4-Dimethane sulphonoxy butane in arachis oil | 0·5 | Stock | 16 | 25 | 70 |
| 1:4-Dimethane sulphonoxy butane in saline | 2·0 | C57 | 5 | 5 | 40 |
| 1:6-Dimethane sulphonoxy hexane in arachis oil | 2·0 | Stock | 5 | 80 | 98 |
| *bis*-*p*-Toluene sulphonoxy-ethyl aniline in arachis oil | 20 | ,, | 12 | 58 | 21 |
| 1:2:5:6-Diepoxyhexane in arachis oil | 5·0 | ,, | 12 | 100 | 50 |
| 1:2:3:4:-Diepoxybutane in arachis oil | 2·5 | ,, | 12 | 100 | 91 |
| 3:4-Benzpyrene in aqueous caffeine (2 per cent) | 0·05 | C57 | 10 | 0 | — |

Examples of greying produced by nitrogen and sulphur mustards are shown in Fig. 1 and 2. The data in Table I show that intradermal injection of a solution containing 0·002 mg. methyl *bis* (*β*-chloroethyl) amine hydrochloride (HN2) per ml. produced greying in half the treated animals, while injection of a more dilute solution produced the effect in only 10 per cent of animals. Thus the mean effective concentration is about 0·002 mg. per ml., equivalent to $10^{-5}$ M. For the purpose of rough quantitative comparison, the injected solution might be considered to be diluted twice in the skin so that the concentration in the tissue which evokes the effect is 0·001 mg. per ml. or $5 \times 10^{-6}$ M.

TABLE II.—*Greying of Hair of Stock Mice with Different Doses of X rays.*

| Dose (r). | Number of mice. | Mice affected. (%). | Latent period (days). | Mice affected on opposite side of body (%). | Latent period (days). |
|---|---|---|---|---|---|
| 50 | 2 | 0 | — | — | — |
| 100 | 2 | 0 | — | — | — |
| 200 | 2 | 0 | — | — | — |
| 250 | 4 | 0 | — | — | — |
| 400 | 2 | 0 | — | — | — |
| 500 | 6 | 33 | 60 | — | — |
| 800 | 6 | 50 | 188 | 50 | 213 |
| 1000 | 6 | 100 | 17 | 33 | 90 |
| 1500 | 2 | 100 | 40 | 100 | 120 |
| 1600 | 2 | 100 | 33 | 100 | 151 |
| 2000 | 1 | 100 | 40 | — | — |

The minimum dose of X rays which produced white hair in all cases (of Table II) was 1000 r (Fig. 3) and the median effective dose about 800 r. Thus irradiation with 800 r produces the same effect of depigmentation as $5 \times 10^{-6}$ M nitrogen mustard (HN2), so that in this reaction 100 r irradiation is equivalent to $0{\cdot}6 \times 10^{-6}$ M nitrogen mustard (HN2). In other radiomimetic effects, such as causing chromosome breaks and killing small animals, the equivalent doses have been considered to be $10^{-6}$ M nitrogen mustard and 100 r, which is of the same order as found with this new effect.

In the case of irradiated mice receiving more than 800 r greying was produced not only at the point of entry of the rays, but a secondary greying was induced at the point at which the rays left the body (Fig. 4). The absorption of radiation in the tissues would be such that the remote side of the body of the mouse would receive something over half of that given to the skin at the point of entry.

TABLE III.—*Greying of Hair Induced by Injection of Water into Mice.*

| Liquid injected. | Strain of mouse. | Number of mice. | Number of sites. | Sites affected (%). | Latent period (days). |
|---|---|---|---|---|---|
| Sterile distilled water | CBA | 12 | 48 | 8 | 40 |
| | FAKI | 6 | 24 | 33 | 30 |
| | C57 | 4 | 16 | 25 | 20 |
| | B | 67 | 228 | 24 | 20 |
| 0·9% sodium chloride | B | 40 | 160 | 0 | — |
| 0·9% sodium chloride acidified ph3 | Stock | 6 | 6 | 0 | — |
| 1*M* sodium chloride | „ | 6 | 24 | 0 | — |
| 3 *M* sodium chloride | „ | 10 | 10 | 40 | 50 |

Control experiments in the series with nitrogen mustard indicated that injection of water caused greying in a proportion of cases. The comparative effects of distilled water and saline are shown in Table III. Of the 46 mice injected intradermally with saline each at 4 sites, none showed greying, but of 70 mice

TABLE IV.—*Greying of Hair of Mice Injected with Substances which might Liberate Free Radicals and Related Compounds.*

Tetralin hydroperoxide, benzoyl peroxide and α-diphenyl-β-trinitro phenylhydrazyl were dissolved in arachis oil—other substances were in aqueous solution.

| Compound. | Molarity. | Strain of mouse. | Number of mice. | Number of sites. | Sites affected (%). | Latent period in days for appearance of 50% of maximum number of patches. |
|---|---|---|---|---|---|---|
| Hydrogen peroxide | 0·01 | B | 12 | 12 | 0 | — |
| | 0·1 | B and CBA | 34 | 58 | 12 | 54 |
| | 0·2 | B | 8 | 8 | 14 | 45 |
| | 0·5 | B | 2 | 8 | 37 | 19 |
| Hydrogen peroxide and ferrous sulphate | 0·01 | B | 5 | 5 | 20 | 50 |
| | 0·05 | A and CBA | 37 | 148 | 100 | 33 |
| | 0·1 | CBA | 25 | 38 | 32 | 40 |
| Ferrous sulphate | 0·05 | A and FAK | 10 | 25 | 88 | 30 |
| Ferric chloride | 0·01 | Stock | 10 | 40 | 60 | 27 |
| | 0·05 | ,, | 10 | 40 | 35 | 27 |
| Ascorbic acid and hydrogen peroxide | 0·01 | B | 8 | 8 | 12 | 44 |
| | 0·1 | C57 | 9 | 18 | 33 | 30 |
| Ascorbic acid | 0·01 | B | 8 | 8 | 12 | 40 |
| | 0·1 | C57 | 9 | 18 | 16 | 30 |
| Hydrogen peroxide and ultra-violet light | 0·5 | Stock | 12 | 48 | 25 | 23 |
| Ultra-violet light | — | ,, | 12 | 12 | 8 (slight) | 9 |
| Hydroxylamine and ferrous ammonium sulphate | 0·05 | ,, | 12 | 24 | 8 | 18 |
| Hydroxylamine | 0·05 | ,, | 12 | 12 | 8 | 32 |
| Ferrous ammonium sulphate | 0·05 | ,, | 12 | 12 | 41 | 20 |
| *tert.* Butyl hydroperoxide | 0·27 | ABC and stock | 27 | 108 | 51 | 25 |
| | 0·55 | Stock | 13 | 40 | 50 | 40 |
| Perbenzoic acid | 0·07 | C57 | 13 | 52 | 81 | 30 |
| Tetralin hydroperoxide | 0·036 | B | 7 | 7 | 0 | — |
| Benzoyl peroxide | 0·02 | B | 8 | 32 | 6 | 40 |
| | 0·04 | B | 6 | 6 | 1 | — |
| Diphenyl *bis* (diazonium hydroxide) | 0·01 | Stock | 8 | 32 | 32 | 26 |
| Benzene diazonium hydroxide | 0·02 | C57 | 15 | 60 | 81 | 60 |
| Sodium-*p*-benzene sulphonate diazonium hydroxide | 0·02 | Stock | 6 | 24 | 0 | — |
| α-Diphenyl-β-trinitro phenyl-hydrazyl | 0·01 | FAK IX | 5 | 20 | 0 | — |
| Azo-*bis*-isobutyronitrile in saline | 0·03 | B | 5 | 20 | 5 | 22 |

injected intradermally with distilled water, greying was produced at 19 per cent of the sites. The results suggest that the cellular change which causes the greying can result from exposure to hypotonic or hypertonic solutions.

Because the effects of radiation may be produced by liberation of free hydroxyl radicals within the cells, a number of reagents which might produce free radicals

have been tested (Table IV). Free hydroxyl radicals can be produced by the interaction of hydrogen peroxide and reducing agents. The first agent of this type to be used was Fenton's reagent, made by mixing hydrogen peroxide and ferrous sulphate, which react together very rapidly. Because of the rapidity of reaction the solutions were injected separately. The solution of ferrous sulphate (0·05 ml.) was injected and then an equal volume of hydrogen peroxide injected at the same site. The injection of hydrogen peroxide alone induced about the same degree of greying as injection of water, but the injection of ferrous sulphate either with or without hydrogen peroxide caused greying in most cases. The induction of the effect with ferrous sulphate and hydrogen peroxide suggested

TABLE V.—*Greying of Hair of Mice Injected with Various Compounds, including Carcinogens (C) and Substances which produce Mutations (M), Chromosome Damage (D) or Blisters (B).*

| Compound. | Solvent. | Concentration (mg./ml.). | Strain of mouse. | Number of mice. | Number of sites. | Sites affected. (%). | Latent period (days). |
|---|---|---|---|---|---|---|---|
| Benzidine (C) | N/40 HCl | 1·8 | Stock | 6 | 24 | 20 | 25 |
| 3:3′-Dihydroxybenzidine (C) | Arachis oil | 10 | „ | 35 | 140 | 6 | 78 |
| | Water | 35 | B | 10 | 40 | 12·5 | 26 |
| 2′-Chloro-4-dimethylaminostilbene (C) | Arachis oil | 12·5 | CBA | 10 | 40 | 0 | — |
| 2′-Fluoro-4-dimethylaminostilbene (C) | Ditto | 50 | CBA | 9 | 36 | 0 | — |
| 4′-Fluoro-4-dimethylaminostilbene (C) | „ | 25 | B | 11 | 44 | 2 | 113 |
| 2-Amino-1-naphthol (C) | „ | 10 | B | 5 | 20 | 10 | 120 |
| Benzoquinone (C) | Water | 0·8 | ABC | 8 | 8 | 62 | 69 |
| Beryllium chloride (C) | „ | 50 | C57 | 7 | 28 | 0 | — |
| | | 10 | C57 | 8 | 32 | 0 | — |
| Sodium arsenite (C) | „ | 1·0 | C57 | 8 | 32 | 0 | — |
| Urethane and ultra-violet light (C) | „ | 100 | Stock | 11 | 48 | 12 | 30 |
| | „ | 50 | „ | 4 | 16 | 8 | 28 |
| Urethane (C) | „ | 100 | Mixed | 5 | 20 | 5 | 52 |
| | „ | 50 | „ | 15 | 60 | 3 | 52 |
| Formaldehyde (M) | „ | 2·0 | C57 | 19 | 76 | 52 | 63 |
| Pyridine-3-diethylcarbonamide (B) | „ | 5·0 | B | 9 | 36 | 19 | 13 |
| Aminopterin (D) | „ | 0·5 | B | 8 | 8 | 0 | — |
| Streptomycin (M ?) | „ | 50 | B | 5 | 5 | 0 | (Kept 48 days) |
| | „ | 25 | B | 5 | 5 | 0 | (Kept 48 days) |
| Cortisone | Saline | 10 | B | 5 | 20 | 0 | — |
| β-Propiolactone (B) | „ | 0·75 | C57 | 10 | 40 | 0 | — |
| Lanthanum acetate | Water | 20 | FAK IX | 4 | 16 | 87 | 40 |

that free hydroxyl radicals might be the cause of the change, but the action might be due to the iron salt alone acting as catalysis of oxidation processes possibly involving free radicals, or to direct chemical combination with some cell constituent such as nucleic acid.

Exposure of mice injected with hydrogen peroxide to ultra-violet light (5 minutes' exposure at 3 cm. distance from an Osira lamp) did not cause a significant increase in the incidence of greying. The penetration of the ultra-violet light was probably insufficient to produce free hydroxyl radicals from the injected peroxide. The injection of urethane with or without exposure to ultra-violet light also had no significant greying effect (Table V).

Combined treatment with hydroxylamine and ferrous ammonium sulphate was tested because the reagents might react to produce free amine ($NH_2$) radicals. The hydroxylamine appeared to reduce the effect of the iron salt.

Of the organic peroxides tested *tert.* butyl hydroperoxide and perbenzoic acid were effective. Of these *tert.* butyl hydroperoxide is known to produce free radicals and has produced mutations in *Neurospora* (Dickey, Cleland and Lotz, 1949).

Of other substances which might yield free radicals listed in Table IV, benzene diazonium hydroxide caused considerable greying. On the other hand, sodium diphenyl *bis* diazonium hydroxide (diazotised benzidine) and sodium *p* benzene sulphonate diazonium hydroxide (diazotised sulphanilic acid) were inactive. The inactivity of these two diazo compounds may be due to inability to penetrate to the cell nucleus before they have reacted or decomposed.

Of the substances in Table V which were inactive, some have been described as being carcinogenic, including the dimethyl-aminostilbene derivatives, which are almost insoluble in water (Haddow, Harris, Kon and Roe, 1948) and 3:3′-dihydroxy-benzidine (Baker, 1950), while others which are mitotic poisons include aminopterin (Dustin, 1950) and $\beta$-propiolactone. Other inactive substances were pyridine-3-diethylcarbonamide which has been described as vesicant (Carlsson and Serin, 1949), cortisone which like aminopterin has a leucopenic action, and streptomycin which may increase the incidence of mutations in bacteria.

The activity of formaldehyde is of interest in view of the reported mutagenic action of this substance (Auerbach, 1950; Rapaport, 1946). Benzoquinone, which caused greying, has been described as carcinogenic (Takizawa, 1940). The benzyl ether of hydroquinone which causes leucoderma in negroes (Schwartz, Oliver and Warren, 1940) and 3:4-dimethylphenylthiourea which inhibits phenoloxidases (Jaques, 1950) did not cause greying. The 2-amino-1-naphthol, a tumour metabolite of $\beta$-naphthylamine described as carcinogenic by Hueper (1938), produced slight greying when injected in oil.

The greying was also induced by application of cold in the form of carbon dioxide snow. When small pieces of carbon dioxide were held on the fur of mice no effect was produced. The application of the carbon dioxide snow to the skin of plucked mice for two minutes produced white patches in each of five mice, within 19 days of the application. Taylor (1949) had observed a similar effect on melanophores of rats, and Berenblum (1930) induced tumours in mice by this treatment.

The loss of colour of the hair might be due to deficiency of one or more enzymes necessary for melanin formation in the skin. For this reason dihydroxyphenyl-alanine ("dopa"), an intermediate in the process, was injected intradermally into white patches induced by nitrogen mustard to see whether it could reverse the process. Five black mice bearing clear white patches were used. The sizes

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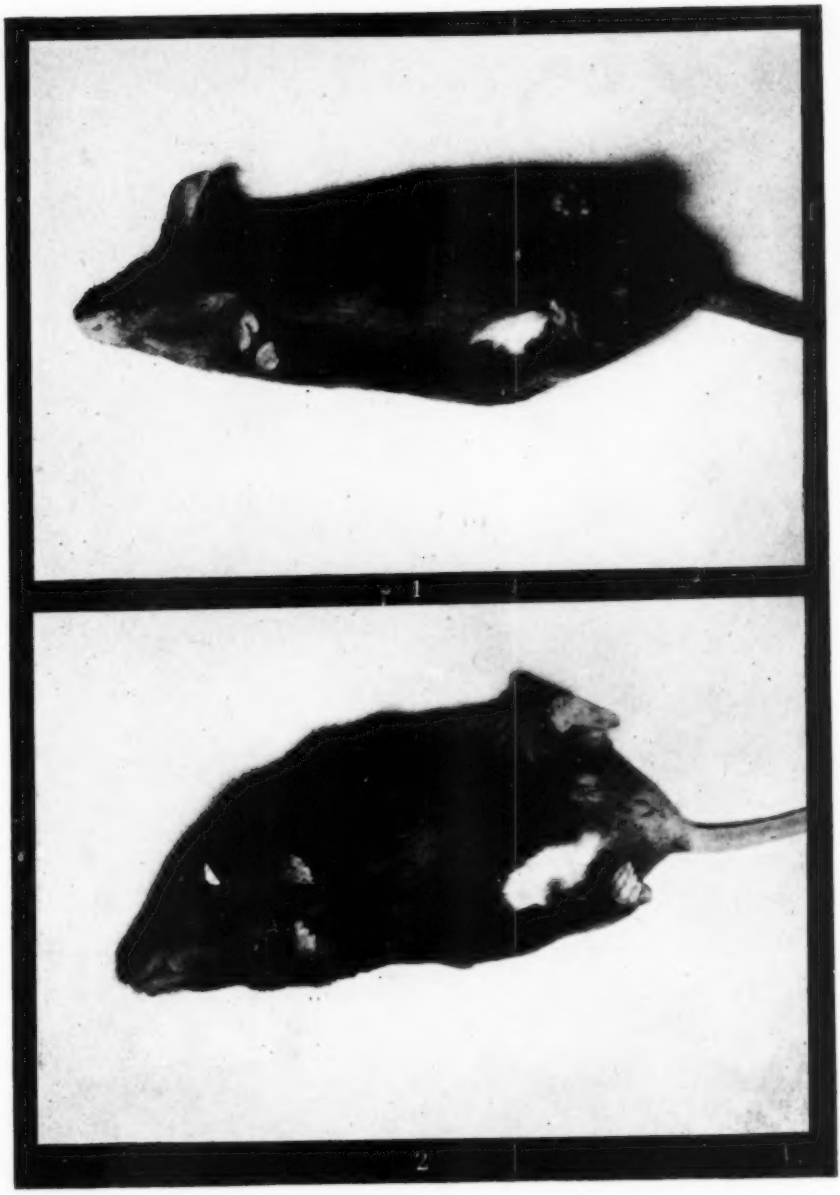
EXPLANATION OF PLATES.

FIG. 1.—Black Stock mouse, 4 months after a single intradermal injection of HN2 (0·1 mg./kg.). The white hairs appeared 3 weeks after injection.

FIG. 2.—Black Stock mouse, 10 months after a single intradermal injection of $\beta:\beta'$-dichloro-diethyl sulphide (4 mg./kg. mustard gas). In this mouse also the white hairs appeared three weeks after injection.

FIG. 3.—Black Stock mouse, 2 months after local irradiation with 1000 r.

FIG. 4.—Black Stock mouse (photographed on mirror so that underside can be seen) 10 months after irradiation with 1600 r, showing white patches at points of entry and exit of X rays. The primary patch on the back appeared after 2 months.



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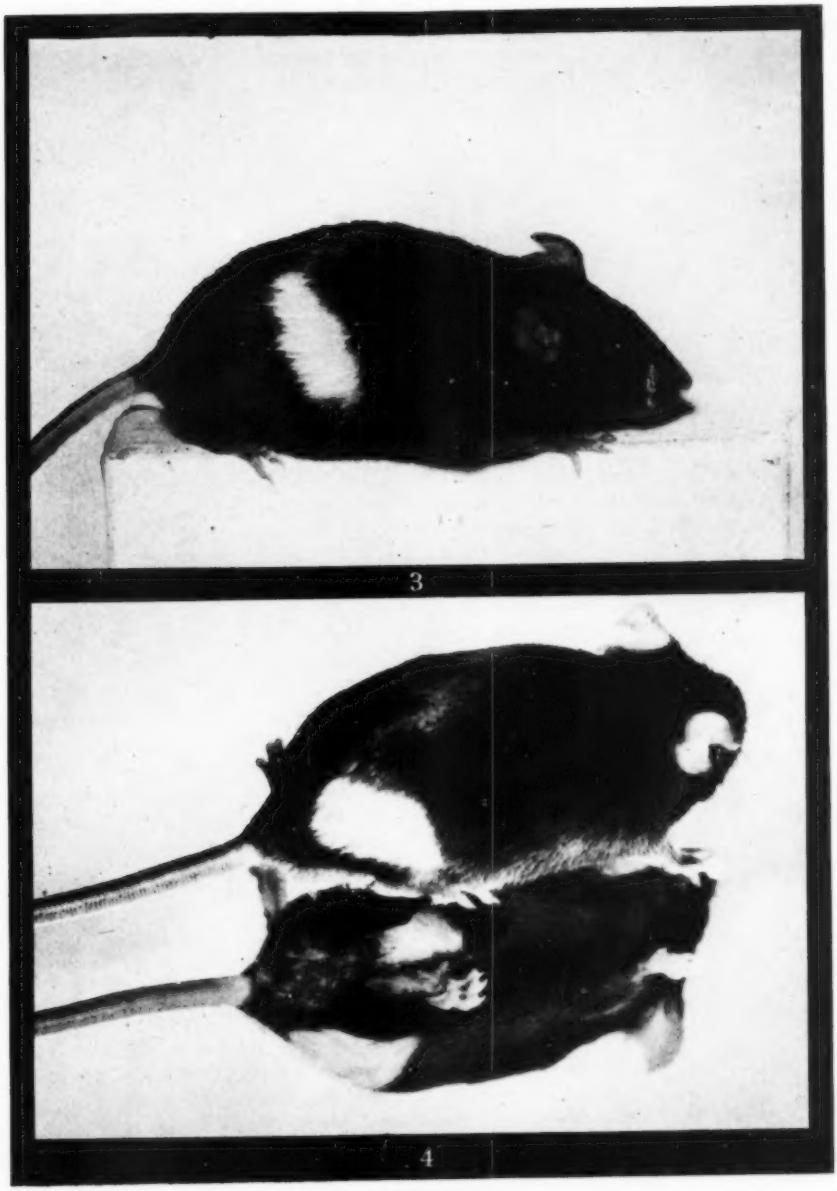

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and shapes of the patches were noted, and then all the white hair and some of the surrounding black hair was plucked out. The mice were injected with 2 per cent " dopa " solution the following day when the hair should be in an actively growing phase. After 10 days the white hair could be seen growing again, and in each case it eventually showed the same distribution as before.

The injection of nitrogen mustard into the skin of coloured guinea-pigs produced scars, the surrounding edge of which had white hairs. This is comparable with the scar production seen after irradiation of guinea-pigs by Frederic (1949) but since he used white animals, there is no record of colour change. A black rabbit was also injected with nitrogen mustard, but no greying was seen in this animal, although it showed scarring similar to that in the guinea-pigs.

## DISCUSSION.

The greying of hair in mice is a radiomimetic effect, the significance of which is difficult to assess. The production of the change by injection of distilled water suggests that the effect of hypotonic solutions on induction of chromosome abnormalities, mutations and other changes should be examined. Although the change is produced by water-soluble carcinogenic agents, there are some substances which have produced greying which have not as yet been shown to be carcinogenic. But as it is hoped that the effect might be used to reveal hitherto unsuspected carcinogenic stimuli, some of these agents are being tested for carcinogenic action. The process of carcinogenesis is obviously complex, and some substances may only have the initiating action and none of the developing or co-carcinogenic action.

Some chemically reactive substances, such as the mineral acids and trichloracetic acid kill cells so that specific biological changes are not produced by such agents, but only by substances with limited toxic action. Although some vesicants are mutagenic and carcinogenic, lewisite, which is a powerful vesicant, does not appear to be mutagenic, and many carcinogens are not vesicant, so that the effects are not always associated. The association between induction of mutations and induction of cancer is much closer.

The effect of local greying resembles the induction of specific chromosome abnormalities, of mutations of cancer and of blisters on human skin in that it is produced by agents with limited toxic action. In the case of the agents producing chromosome changes, mutations or cancer the effect is limited to specific parts of cells. In the local greying of hair the effect is restricted to certain specific cells of the tissue.

## SUMMARY.

(1) The intradermal injection of nitrogen mustards or sulphur mustard into coloured mice causes a permanent greying or bleaching of the hair similar to that occurring after exposure to ionising radiations. This greying appears to be a radiomimetic effect.

(2) Similar greying of hair occurs in a proportion of cases when water is injected but never following the injection of saline.

(3) Greying of hair was produced by injection of (*a*) known carcinogens, such as *β*-naphthyl-*bis* (*β* chloroethyl) amine and butadiene diepoxide, the mustards, 2:4:6-tri(ethyleneimino) 1:3:5-triazine and benzoquinone, (*b*) known cytotoxic

agents and mutagens which are not yet known to be carcinogenic, such as *tert.* butyl-hydroperoxide and formaldehyde, (*c*) substances which are not known to be carcinogenic, cytotoxic or mutagenic, including salts of iron and lanthanum and substances which might give free radicals, such as benzene diazonium hydroxide and perbenzoic acid.

(4) Greying was not produced by injection of some carcinogens, including urethane and dimethylaminostilbene derivatives.

(5) This effect, which may be an induced permanent variation due to preferential lethal action on pigment-producing dendritic cells, is an example of limited toxic action, and so allied to vesication and induction of mutations and cancer.

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# THE REACTIVITY OF AROMATIC BONDS, WITH REFERENCE TO CARCINOGENIC COMPOUNDS OF 1 : 2-BENZANTHRACENE.

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Most of the chemical properties characteristic of conjugated systems are ascribed to the unsaturation or $\pi$ electrons whose wavefunctions are antisymmetric with respect to the plane of the molecular framework. This symmetry property of the wavefunctions permits the $\pi$ electron system to be treated independently of the other electrons in the system except in so far as interaction terms are implicit in the potential field of the Hamiltonian operator for each $\pi$ electron.

Of the two methods generally available for the quantum mechanical treatment of $\pi$ electron systems, the molecular orbital (M.O.) method has proved more readily applicable to the problems of chemical reactivity. Methods based on the valence bond (V.B.) description, due originally to Pauling (1933), have been given by Daudel and Daudel (1948), and Pullman and Pullman (1949) as developments of a similar procedure proposed by Svartholm (1942). There remains, however, a measure of arbitrariness in these methods which places some doubt on the meaning and adequacy of the results (Moffitt, 1949).

Two different methods of approach within the framework of the M.O. theory have been used for the discussion of chemical reactivity. The first, due originally to Wheland (1942), calculated the energy of an assumed $\pi$ electron configuration for the transition state, in which the requisite number of electrons appropriate to a given type of reaction were assumed localised at the site of reaction. The variation of activation energy for reaction at different positions in the molecule was then attributed to the different energies required to localise the electrons. It was found that these different calculated localisation energies could be satisfactorily correlated with the experimental rates of reaction for the different types of chemical reactivity.

The second M.O. method is based upon correlations between quantities describing the $\pi$ electron configuration in the non-reacting ground state and rates of reaction. The present paper is concerned with the application of this method to the discussion of the reactivity of the 3–4 bond of derivatives of 1 : 2-benzanthracene in the light of the experimental work of Badger (1950) and his associates on the reactions of osmium tetroxide with these compounds. Perturbation methods used to deduce the $\pi$ electron configuration of a derivative from that of the parent hydrocarbon will facilitate the comparison of results obtained theoretically with experimental evidence. We shall see that some of the conclusions reached about the reactions of the 3–4 bond in this particular series may, with due caution, be regarded as rather typical of the reactions of aromatic bonds in general.

In the first place, therefore, we shall present an outline of the method to be used, followed by a brief discussion of some of the present theoretical interpretations of the reactions of aromatic systems. The experimental results for the derivatives of 1 : 2-benzanthracene will then be considered in terms of those obtained theoretically, and we shall see to what extent these are compatible with the above interpretations. Finally we shall discuss the application of the results to the theories which have been put forward to explain the carcinogenic activity of some of the derivatives of 1 : 2-benzanthracene.

## THE M.O. METHOD.

We shall adopt the notation and method of Coulson and Longuet-Higgins (1947*a*, *b* ; 1948*a*, *b*) in their treatment of the M.O. theory of conjugated systems. For simplicity we shall deal with alternant hydrocarbons and their derivatives containing an even number of $\pi$ electrons (the extension to odd-numbered systems is quite straightforward). It will be necessary moreover to omit most of the fundamental theory, and generally we shall quote just those results relevant to the present discussion.

The M.O. method describes the electronic structure in terms of one-electron wavefunctions, $\psi$. The customary approximation takes $\psi$ to be a linear combination of the atomic $2p_z$ orbitals $\phi$ so that

$$\psi = \sum_{r=1}^{n} c_r \phi_r, \quad . \quad . \quad . \quad . \quad . \quad . \quad (1)$$

where the $c_r$ are numerical coefficients and $n$ is the number of conjugated atoms. The determination of the permitted values of the energy $E$ and of the corresponding wavefunctions $\psi$ by solution of the secular equations is familiar. Where necessary the different $\psi$ are distinguished by an index, $j$ :

$$\psi_j = \sum_{r=1}^{n} c_{rj} \phi_r. \quad . \quad . \quad . \quad . \quad . \quad . \quad (2)$$

The electron distribution in the ground state is described in terms of :

(i) the charge density $q_r$ at atom $r$ defined by

$$q_r = 2 \sum_{j=1}^{\frac{n}{2}} c_{rj}^2 \quad . \quad . \quad . \quad . \quad . \quad . \quad (3)$$

(ii) the bond order $p_{st}$ of the partial double bond joining atoms $s$ and $t$ defined by

$$p_{st} = 2 \sum_{j=1}^{\frac{n}{2}} c_{sj} c_{tj}, \quad . \quad . \quad . \quad . \quad . \quad . \quad (4)$$

where the summations are taken over the lowest $n/2$ occupied orbitals.

A further quantity, the free valence gives a measure of the available bonding power of an atom. The total bonding assumed by the carbon atom $r$ may be expressed as

$$N_r = 3 + \sum_{s} p_{rs}, \quad . \quad . \quad . \quad . \quad . \quad (5)$$

in which the summation is taken over all atoms $s$ which are neighbours to $r$ and the number 3 represents the bonding of the three $\sigma$ bonds. It has been shown that the maximum value of $N_r$ is 4·732 (Burkitt, Coulson and Longuet-Higgins, 1951), which has led to the definition of the free valence at the carbon atom $r$ as

$$F_r = 4{\cdot}732 - N_r. \quad . \quad . \quad . \quad . \quad . \quad (6)$$

These theoretical quantities $q_r$, $p_{st}$ and $F_r$ have been correlated in various ways, to be discussed later, with chemical reactivity.

Alternative expressions have been given for $q_r$ and $p_{st}$ in terms of the minors of the secular determinant. Thus

$$q_r = 1 - \frac{1}{\pi}\int_{-\infty}^{+\infty} \frac{\Delta_{r,r}(iy)}{\Delta(iy)}\, dy, \quad . \quad . \quad . \quad . \quad (7)$$

$$p_{st} = \frac{1}{\pi}\int_{-\infty}^{+\infty} \frac{\Delta_{s,t}(iy)}{\Delta(iy)}\, dy, \quad . \quad . \quad . \quad . \quad (8)$$

in which $\Delta a,b$ represents the minor obtained by crossing out the $a^{\text{th}}$ row and the $b^{\text{th}}$ column of the secular determinant $\Delta(iy)$. The numerators and denominanators of the integrands in (7) and (8), though functions of $(iy)$, are polynominals in either even or odd powers of $y$, and the integration is along an axis containing no singularities (except when $n$ is odd—a case dealt with by Coulson and Longuet-Higgins (1947*a*)).

Now the secular equations are solved in terms of two types of parameters $\alpha_r$ the coulomb integral for atom $r$ and $\beta_{st}$ the resonance integral for the bond $st$. It is customary to fix the zero of energy by putting $\alpha_c$ for a carbon atom equal to zero, and to consider all $C - C$ resonance integrals to be equal, denoted by $\beta$, say. Then the coulomb integral for any heteroatom $X$ is written in terms of the resonance integral $\beta$ as

$$\alpha_x = \alpha_c + k\beta, \quad . \quad . \quad . \quad . \quad . \quad (9)$$

where $k$ is a number. Since $\beta$ is negative, $k$ is positive or negative according as the heterosystem $X$ is more or less electronegative than carbon.

The solutions of the secular equations may be used to obtain the electron configuration in terms of $q_r$, $p_{st}$ and $F_r$ for both pure hydrocarbons and molecules containing heterosystems. It is merely necessary to put $\alpha_c = 0$ for all sites occupied by carbon atoms and to choose appropriate values of $\alpha_x$ (i.e., of $k$) for the heterosystems. Some authors have further assumed a heterosystem to modify also both the coulomb integrals of the adjacent carbon atoms by an inductive effect and the resonance integrals of the adjacent bonds (Longuet-Higgins and Coulson, 1947 ; Wheland and Pauling, 1935). Since these refinements do not modify the main conclusions to be reached for the theoretical interpretation of bond reactions in the systems examined, we shall limit the present discussion to the simple treatment whereby a heterosystem may be represented merely by a change in coulomb integral at the position occupied by the system. This then facilitates the use of perturbation theory for the determination of the electron distribution for a derivative in terms of that of

the parent hydrocarbon, and obviates the solution of the secular equations for each value $k$ of (9) representing different heterosystems.

This procedure is strictly legitimate for aza derivatives in which the nitrogen atom supplies just one $\pi$ electron like the carbon atom it replaces, and in which the molecular orbitals are analogous to those of the parent hydrocarbon. Methyl substitution is, however, more complicated if hyperconjugation is assumed to take place. Mulliken, Rieke and Brown (1941) have shown that the hyperconjugation problem can be fitted into the general M.O. scheme by treating the $H_3$ group as a single pseudo atom $X$ so that for $R-C\equiv H_3$ we may write $R-C-X$, in which $R-H$ is the unsubstituted conjugated system with its original number of $\pi$ electrons, and where $C$ and $X$ are each supposed to contribute one additional $\pi$ electron. The molecular orbitals now embrace the whole system $R-C-X$. One must now choose appropriate values for the coulomb integrals for atoms $C$ and $X$ and resonance integrals for bonds $R-C$ and $C-X$. V. A. Crawford (unpublished work) has investigated the particular case of hyperconjugation in toluene, and has chosen the above parameters to fit the experimentally observed dipole moment and simultaneously the charge distribution in the ring required by the evidence of the chemical reactivity of toluene. We are not primarily interested in the present work in the detailed mechanism of methyl hyperconjugation, but rather in the modification of the charge distribution of the original unsubstituted system. It can be shown that this modification can be reproduced with an accuracy sufficient for the purposes of the present work by representing the $CH_3$ group by a change in the coulomb integral of the carbon atom to which it is attached. This change must correspond to a decrease in its electronegativity, so that $k$ of equation (9) must be negative (for nitrogen it is positive). The perturbation technique to be described in the next section may then be applied to the case of methyl substitution.

In passing it may be added that we have shown in a limited number of calculations that the modification of the charge distribution of a parent hydrocarbon due to conjugation with a substituent $X$ which supplies two $\pi$ electrons to the system, e.g., $X=OH$, $NH_2$, etc., can also be well reproduced by assuming merely a perturbation of the coulomb integral of the adjacent carbon atom. This modification of charge distribution implies, of course, changes in charge density, bond order and free valence as described in the next section.

*Perturbation formulae*

For the heterosystem at position $u$ the changes in charge density at atom $r$ and of bond order for the bond $st$ may be written

$$\delta q_r=\frac{\partial q_r}{\partial \alpha_u}\delta\alpha_u+\tfrac{1}{2}\frac{\partial^2 q_r}{\partial \alpha_u^{\,2}}\delta\alpha_u^{\,2}+\ldots, \quad . \quad . \quad . \quad (10)$$

$$\delta p_{st}=\frac{\partial p_{st}}{\partial \alpha_u}\delta\alpha_u+\tfrac{1}{2}\frac{\partial^2 p_{st}}{\partial \alpha_u^{\,2}}\delta\alpha_u^{\,2}+\ldots, \quad . \quad . \quad . \quad (11)$$

where the heterosystem is represented by a coulomb integral $\alpha_u$ given by

$$\alpha_u=\alpha_c+\delta\alpha_u, \quad . \quad . \quad . \quad . \quad . \quad (12)$$

which by comparison with (9) gives

$$\delta\alpha_u=k\beta, \quad . \quad . \quad . \quad . \quad . \quad (13)$$

The first order perturbation coefficients of equations (10) and (11) have been termed the mutual atom and atom-bond polarisabilities respectively. It can be shown, however, that the second term on the right-hand side of (10) and the first of (11) are zero. In conformity with present usage, we shall adopt the following notation for the coefficients

$$\pi_{r,u} = \frac{\partial q_r}{\partial \alpha_u} \quad ; \quad \pi'_{r,u} = \tfrac{1}{2} \frac{\partial^2 q_r}{\partial \alpha_u^2} = 0, \quad . \quad . \quad (14)$$

$$\pi_{st,u} = \frac{\partial p_{st}}{\partial \alpha_u} = 0 \quad ; \quad \pi'_{st,u} = \tfrac{1}{2} \frac{\partial^2 p_{st}}{\partial \alpha_u^2}. \quad . \quad . \quad . \quad (15)$$

These polarisability coefficients may then be regarded as giving the perturbed charge densities and bond orders to the second order, valid to the second power in $k$.

Coulson and Longuet-Higgins (1947*a*) have derived expressions for $\pi_{r,u}$ for alternant hydrocarbons both in terms of the coefficients $c_{rj}$ of the unperturbed wavefunctions of equation (2) and by differentiation of equation (7).

The $\pi'_{t,u}$ may be obtained by extending these methods. The results are summarised in the following pairs of alternative formulae:

$$\pi_{r,u} = 4 \sum_{j=1}^{\frac{n}{2}} \sum_{k=\frac{n}{2}+1}^{n} \frac{c_{uj}\, c_{rj}\, c_{uk}\, c_{rk}}{E_j - E_k}, \quad . \quad . \quad . \quad (16)$$

$$\pi_{r,u} = \frac{1}{\pi} \int_{-\infty}^{+\infty} \left[ \frac{\Delta_{r,u}(iy)}{\Delta(iy)} \right]^2 dy, \quad . \quad . \quad . \quad . \quad (17)$$

$$\pi'_{st,u} = 2 \sum_{j=1}^{\frac{n}{2}} \left\{ c_{sj}{}^{I} c_{tj}{}^{I} + c_{sj} c_{tj}{}^{II} + c_{sj}{}^{II} c_{tj} - c_{sj} c_{tj} \sum_{k}{}' \frac{c_{uk}{}^2 c_{uj}{}^2}{(E_j - E_k)^2} \right\}, \quad . \quad (18)$$

in which

$$c_{sj}{}^{I} = \sum_{k}{}' \frac{c_{uk} c_{uj}}{E_j - E_k} \; . \; c_{sk},$$

and

$$c_{sj}{}^{II} = \sum_{k}{}' \left\{ \frac{(c_{uk}{}^2 - c_{uj}{}^2)_u c_k c_{uj}}{(E_j - E_k)^2} + \sum_{l}{}'' \frac{c_{ul}{}^2 c_{uj} c_{uk}}{(E_j - E_k)(E_j - E_l)} \right\} c_{sk},$$

where $\sum_{k}{}'$ and $\sum_{l}{}''$ mean that $k = j$ and $k, l = j$ are to be omitted respectively.

$$\pi'_{st,u} = \frac{1}{\pi} \int_{-\infty}^{+\infty} \frac{\Delta_{s,u}(iy) \quad \Delta_{t,u}(iy) \quad \Delta_{u,u}(iy)}{\Delta^3(iy)}. \quad . \quad . \quad (19)$$

The separate minors in the above integral formulae are all functions of $(iy)$, but the integrands themselves are real functions of $y$. The last summation of equation (18) represents a second order normalisation term frequently neglected in the literature (Schiff, 1949).

A formula analogous to (10) and (11) may be written for the perturbed free valence at atom $r$:

$$\delta F_r = \frac{\partial F_r}{\partial \alpha_u}\delta\alpha_u + \tfrac{1}{2}\frac{\partial^2 F_r}{\partial \alpha_u^2}\delta\alpha_u^2 + \ldots \quad (20)$$

From the definition of $F_r$ given by (5) and (6) and from the properties of the bond order perturbation terms it is clear that the first order term in (20) is zero. The non-zero second order term will be written

$$F'_{r,u} = \tfrac{1}{2}\frac{\partial^2 F_r}{\partial \alpha_u^2} \quad (21)$$

An explicit formula for this quantity analogous to (19) has been given (Coulson, 1948), but in the present case the value will be deduced from the available $\pi'_{st,u}$ terms.

*The reactivity of aromatic bonds.*

A review of the reactions of aromatic bonds has recently been given by Badger (1951).

It is necessary to preface this section with a brief description of the significance of $q_r$, $p_{st}$ and $F_r$ to the discussion of the reactivity of aromatic systems.

It can be shown that for heterolytic reactions the change in $\pi$ electron energy $\delta E$ due to the approach of a reactant to the atom position $r$ is given by

$$\delta E = q_r\delta\alpha_r + \pi_{r,r}\delta\alpha_r^2 + \ldots, \quad (22)$$

where $\delta\alpha_r$ is the change in coulomb integral at position $r$ due to the proximity of the reactant. To a first order, therefore, the rate of reaction will depend upon the magnitude of the charge $q_r$ at the position of attack. Equation (22) describes, however, conditions in the early stages of reaction only when the reactant merely polarises the $\pi$ electron system. However, the calculations of Wheland (1942) on assumed transition state complexes nearly always predict the same positions of reactivity, and lend support to the assumption of the above equation (22) as a criterion of heterolytic reactivity. By application to a variety of molecular systems, rates of reaction have been successfully correlated with the corresponding values of $q_r$, whilst for alternant hydrocarbons for which $q_r$ is unity at every atomic position, the reactivity has been correlated with the $\pi_{r,r}$ second order term. (A list of references is given in a comprehensive review by Longuet-Higgins and Wheland, 1950.)

It has been suggested that free valence may largely govern homolytic reactivity. Burkitt, Coulson and Longuet-Higgins (1951) have compared the polarisation energy required to localise one $\pi$ electron at the position of attack with the free valence at the same position for a large number of hydrocarbons. A good correlation is obtained for alternant hydrocarbons. A less satisfactory situation arises in the case of non-alternant hydrocarbons and free radicals. Now it can be shown that the free valences and self-polarisabilities, $\pi_{r,r}$, in a typical series of alternant hydrocarbons vary in a parallel fashion. It must therefore be concluded that for hydrocarbons, free radical reactivity which may be correlated with free valence could equally well be correlated with self-polarisability (since $q_r = 1$ for alternant hydrocarbons). It is not suggested that in all cases free valence and self-polarisability necessarily vary exactly in a parallel fashion.

Calculated bond orders have been correlated successfully with the experimental rates of addition to a bond, so that high values of bond order correspond to highly reactive bonds (Badger, 1950 ; Berthier, Coulson, Greenwood and Pullman, 1948). The most complete set of experimental data on bond reactions has been given by Badger and his collaborators using osmium tetroxide as the bond reagent (Badger, 1949 ; Badger and Reed, 1948 ; Badger and Lynn, 1950). Brown (1949, 1950, 1951) has used the bond localisation energy, defined as "the energy required to perturb the molecule so that the bond in question becomes a double bond," as a criterion of bond reactivity. The successful application of this method in predicting quantitatively, with considerable accuracy, the ratios of rates of addition of osmium tetroxide to bonds in certain aromatic hydrocarbon systems seems to affirm its validity as a criterion of this

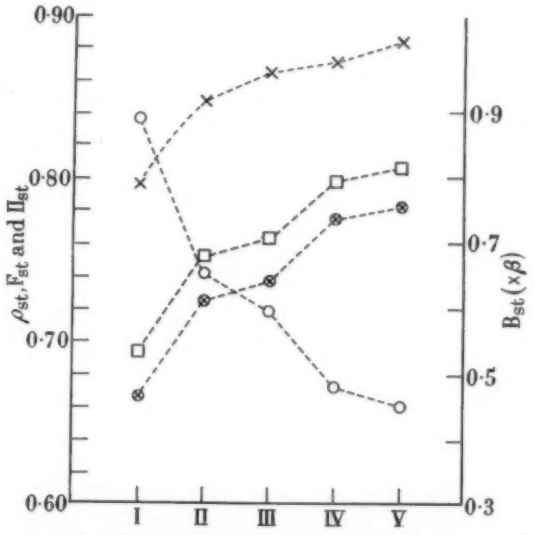



FIG. 1.—I. Benzene bond. II. Naphthalene 1-2 bond. III. Anthracene 1-2 bond. IV. Phenanthrene 9-10 bond. V. Benzanthracene 3-4 bond.

$\otimes \equiv p_{st}$ ; $\square \equiv F_{st}$ ; $\times \equiv \Pi_{st}$ ; $\circ \equiv B_{st}$.

type of reaction. Moreover Brown (1949) has also shown that it is possible to correlate bond order with bond localisation energy at least approximately, suggesting, on the assumption of the latter as the more reliable criterion, that the bond order also should be a useful index of reactivity.

Unfortunately correlations of this kind for hydrocarbons are not unique. In Fig. 1 we show the variations of the calculated quantities

$p_{st}$

$\Pi_{st} = \pi_{s,s} + \pi_{t,t}$

$F_{st} = F_s + F_t$

$B_{st}$ = bond localisation energy

for a system of comparable bonds for which the experimental rates of addition of osmium tetroxide increase in the order

benzene < naphthalene 1-2 bond < anthracene 1-2 bond

< phenanthrene 9-10 bond < 1-2 benzanthracene 3-4 bond

The above three quantities, $p_{st}$, $II_{st}$ and $F_{st}$, were chosen to represent, perhaps very roughly, possible criteria for the addition of osmium tetroxide on the assumptions, respectively, that the reaction depends in some way on the charge density in the bond, that it is heterolytic in nature dependent on an equation of the type (22), and that it is homolytic. Suffice it to say that for aromatic hydrocarbons it is possible to correlate the experimental bond reactivity with a number of chosen theoretical quantities which do not permit one to determine the nature of the reaction unambiguously.

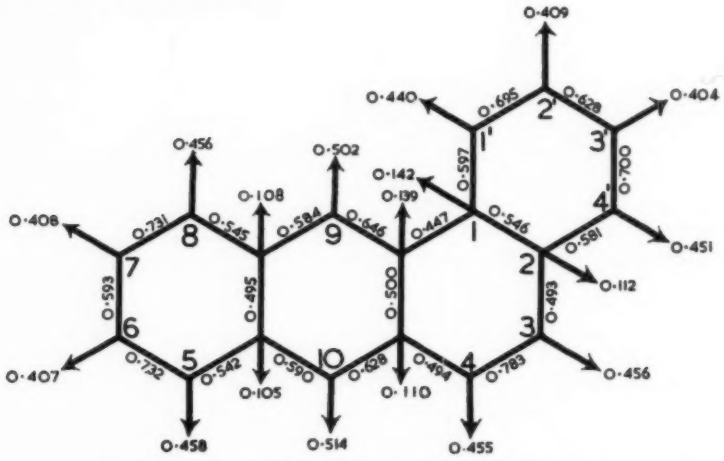



FIG. 2.—1 : 2-Benzanthracene-bond orders and free valences.

We shall now show that the treatment of substituted molecules helps to resolve the problem, at least in part. We propose to examine the $\pi$ electron configuration of substituted 1 : 2-benzanthracene in the light of experimental results of Badger on the rate of addition of osmium tetroxide to the 3-4 bond (Badger and Lynn, 1950 ; Badger, 1950).

*The 3-4 bond of benzanthracene derivatives.*

The $\pi$ electron configuration in the region of the 3-4 bond for benzanthracene derivatives will be determined from that of the parent molecule by means of the perturbation formulae (10) (11) and (20) in which the perturbation coefficients $\pi_{r,u}$, $\pi'_{st,u}$ and $F'_{r,u}$ are calculated from the formulae (17) and (19) using methods of numerical integration (Coulson and Jacobs, 1949 ; Bickley, 1939).

The calculations involved in the application of (18) to the benzanthracene system are far too cumbersome to be of practical value. Equation (16) has,

however, been used to provide a check on the results of (17). Both (16) and (18) have been applied to simpler systems.

The changes in the quantities describing the $\pi$ electron configuration due to a single substituent at position $u$ may be written to the second order

$$\delta q_r = \pi_{r,u}\delta\alpha_u \quad = \pi_{r,u} \times k\beta,$$
$$\delta p_{st} = \pi'_{st,u}\delta\alpha_u{}^2 = \pi'_{st,u} \times k^2\beta^2,$$
$$\delta F_r = F'_{r,u}\delta\alpha_u{}^2 = F'_{r,u} \times k^2\beta^2.$$

The $\pi$ electron configuration for the parent molecule 1 : 2-benzanthracene is given in Fig. 2, in which the bond orders are placed alongside the corresponding bonds and the free valences are denoted by figures attached to the arrows. The $q_r$ are all equal to unity. It will be noted that on the assumption that bond order and free-valence are criteria of reactivity, the region of the 3-4 bond would be expected to be highly reactive. On the other hand the self-polarisabilities given in Fig. 3 predict a similar result.

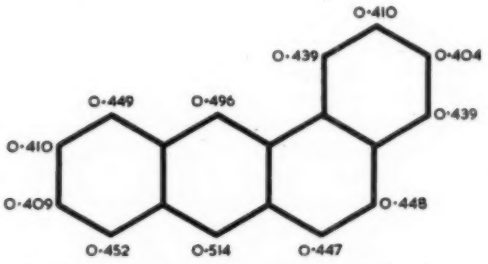



FIG. 3.—1 : 2-Benzanthracene-self polarisabilities.

In Table I are given the $\pi_{r,u}$ relating to the two positions 3, 4 and all other positions of the perimeter of 1 : 2-benzanthracene. Similarly Table II gives the $\pi'_{st,u}$ for the bonds 2–3, 3–4 and 4–14. All three are required for the determination of the $F'_{r,u}$ for the atoms 3, and 4, given in Table III.

TABLE I.

| $u$. | $\pi_{3,u} \times \beta$. | $\pi_{4,u} \times \beta$. | $\{\pi_{3,u} + \pi_{4,u}\} \times \beta$. |
|---|---|---|---|
| 1′ | −0·0044 | +0·0033 | −0·0011 |
| 2′ | +0·0092 | −0·0449 | −0·0357 |
| 3′ | −0·0106 | +0·0005 | −0·0106 |
| 4′ | +0·0249 | −0·0488 | −0·0239 |
| 3 | +0·4477 | −0·2835 | +0·1642 |
| 4 | −0·2835 | +0·4473 | +0·1638 |
| 5 | −0·0148 | +0·0057 | −0·0091 |
| 6 | +0·0005 | −0·0036 | −0·0031 |
| 7 | −0·0162 | +0·0035 | −0·0127 |
| 8 | +0·0033 | −0·0027 | +0·0006 |
| 9 | +0·0099 | −0·0126 | −0·0027 |
| 10 | −0·0738 | +0·0351 | −0·0387 |

TABLE II.

| $u$ | $\pi_{22'}, _u \times \beta^2$. | $\pi_{34'}, _u \times \beta^2$. | $\pi_{4\,14'}, _u \times \beta^2$. |
|---|---|---|---|
| 1′ | +0·0002 | −0·0005 | +0·0014 |
| 2′ | +0·0067 | −0·0050 | +0·0010 |
| 3′ | −0·0025 | +0·0007 | −0·0005 |
| 4′ | +0·0176 | −0·0092 | +0·0037 |
| 3 | −0·0503 | −0·0842 | +0·0214 |
| 4 | +0·0212 | −0·0840 | −0·0504 |
| 5 | +0·0013 | −0·0023 | +0·0035 |
| 6 | +0·0003 | −0·0002 | +0·0001 |
| 7 | +0·0007 | −0·0016 | +0·0021 |
| 8 | +0·0008 | −0·0002 | +0·0000 |
| 9 | +0·0047 | −0·0021 | +0·0013 |
| 10 | +0·0091 | −0·0175 | +0·0298 |

TABLE III.

| $u$ | $F_3', _u \times \beta^2$. | $F_4', _u \times \beta^2$. | $\{F_3', _u + F_4', _u\} \times \beta^2$ |
|---|---|---|---|
| 1′ | +0·0003 | −0·0009 | −0·0006 |
| 2′ | −0·0017 | +0·0040 | +0·0023 |
| 3′ | +0·0018 | −0·0002 | +0·0016 |
| 4′ | −0·0084 | +0·0055 | −0·0029 |
| 3 | +0·1345 | +0·0628 | +0·1973 |
| 4 | +0·0628 | +0·1344 | +0·1972 |
| 5 | +0·0010 | −0·0012 | −0·0002 |
| 6 | −0·0001 | +0·0001 | +0·0000 |
| 7 | +0·0009 | −0·0005 | +0·0004 |
| 8 | −0·0006 | +0·0002 | −0·0004 |
| 9 | −0·0026 | +0·0008 | −0·0018 |
| 10 | +0·0084 | −0·0123 | −0·0039 |

TABLE IV.

| | Rate constant $10^3K$. | $\frac{\delta q_3 + \delta q_4}{-k}$. | $\frac{\delta p_{34}}{k^2}$. | $\frac{\delta F_3 + \delta F_4}{k^2}$ |
|---|---|---|---|---|
| 1 : 2-benzanthracene | 0·48 | 0 | 0 | 0 |
| 3-methyl-1 : 2-benzanthracene | 0·50 | −0·1642 | −0·0842 | +0·1973 |
| 6-methyl- ,, | 0·64 | +0·0031 | −0·0002 | +0·0000 |
| 5 : 6-dimethyl- ,, | 0·64 | +0·0122 | −0·0006 | +0·0009 |
| 5 : 7-dimethyl- ,, | 0·80 | +0·0218 | −0·0048 | +0·0002 |
| 2′ : 7-dimethyl- ,, | 0·83 | +0·0484 | −0·0066 | +0·0027 |
| 10-methyl- ,, | 0·91 | +0·0387 | −0·0175 | −0·0039 |
| 9-methyl- ,, | 0·96 | +0·0027 | −0·0021 | −0·0018 |
| 9 : 10-dimethyl- ,, | 2·7 | +0·0414 | −0·0129 | −0·0007 |
| 5 : 6 : 9 : 10-tetra methyl-1 : 2-benzanthracene | 2·9 | +0·0536 | −0·0141 | +0·0025 |

$\triangle Q = \delta q_3 + \delta q_4$; $\triangle F = \delta F_3 + \delta F_4$.
$k$ is negative for $CH_3$.

We may indicate immediately the implications of the results for mono substitution :

(1) Substitution at all positions on the perimeter except $u = 3'$ produces a decrease of bond order of the 3–4 bond for all substituents irrespective of their electronegativity. This follows from the dependence of $\delta p_{st}$ on $\delta\alpha_u^2$.

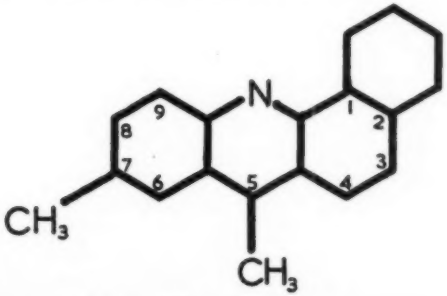
(2) The sum $\pi_{3,u} + \pi_{4,u}$ indicates a net flow of charge into the 3–4 region for an electropositive substituent and a net flow away from the region for an electronegative substituent for all positions except $u = 8, 3, 4$. Also since $q_r = 1$ in the parent hydrocarbon, the charge distribution in the derivative is asymmetric about the value unity at the 3–4 atoms.

(3) There are no similar regularities in the variations of free valence.

The results may be compared with the experimental evidence on the rates of addition of osmium tetroxide to the 3–4 bond of derivatives of 1 : 2-benzanthracene. In Table IV are given the rates of addition for various methyl and dimethyl derivatives (Badger, 1950). The changes in bond order $\delta p_{34}$, in the sums of the free valences $\Delta F$ and of the charge densities $\Delta Q$ are also given in Table IV in terms of the parameter $k$ representing the $CH_3$ group according to equation (13). Since all the substituting groups are the same we need not specify the value of $k$, merely noting that it must be negative. The results for multiple substitution are complicated by the appearance of non-zero cross terms in $\delta p_{st}$ and $\delta F_r$. These cross terms, which have been included in the calculated results of Table IV, are in general much smaller than the corresponding non-cross terms.

It is clear that we cannot correlate increase in bond order of the 3–4 bond with the experimentally determined increase in the rate of addition $K$ of osmium tetroxide to the 3–4 bond, since an increase in reactivity consequent upon methyl substitution is in fact accompanied by a decrease of bond order. Similarly, analysis of the results for the variation of the net free valence $\Delta F$ permits no correlation of this quantity with $K$ (nor is it possible to correlate $K$ with the individual free valence variations $\delta F_3$ and $\delta F_4$). There is, however, a correlation between increase in $K$ and increase in the net charge variation $\Delta Q$ (though not with the individual variations $\delta q_3$ and $\delta q_4$). Substitution at the bond itself gives, not surprisingly, an anomalous result.

Badger (1950) also notes a decrease in the rate of addition of $Os\,O_4$ upon the introduction of a N atom in 5 : 7-dimethyl-1 : 2-benzacridine. This can be explained in terms of the variation of the total charge $\Delta Q$, and not by the corresponding change in either the bond order or the total free valence. Thus the total charge suffers a decrease on nitrogen substitution,

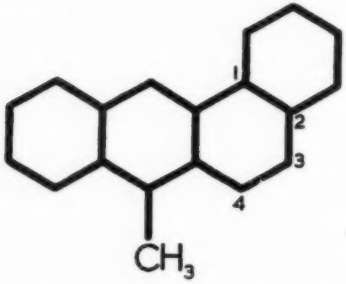


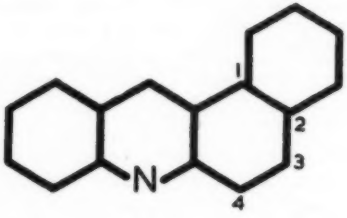

in contrast to the increase due to $CH_3$ substitution.

Now we have seen that for a system of hydrocarbons the rate of addition of Os $O_4$ to certain bonds (the most reactive) can be correlated with bond order, with the sum of the free valences of the two atoms concerned and with the sum of their self-polarisabilities. Furthermore it was noted that for these molecules these theoretical quantities varied in a parallel fashion as one passed from one molecule to another. When, however, this parallel variation no longer holds as in the treatment of the derivatives described above certain of these correlations break down. (Now of course we must consider the first order terms $q_r$ which are no longer unity, rather than the second order self-polarisabilities.) We conclude therefore that bond reactions of this type are not governed largely by bond order (or double bond character), nor by the free valence, but seem to depend upon the charge densities at the atoms concerned. This conclusion is satisfactory in virtue of the direct relation between charge density $q_r$ and energy variations (equation (22)), and suggests that the reaction is heterolytic in nature. The asymmetry of the charge distribution at atoms 3 and 4 suggests that the two positions are attacked consecutively rather than simultaneously, and throws some doubt on the validity of the assumption that the reaction proceeds via an initial step requiring the localisation of a pure double bond.

In spite of the apparently successful correlation of the total charge density with experimental rates of addition to the 3–4 bond it seems likely that the reaction mechanism involved is complicated. The following suggested mechanism may, however, offer a rough outline.

Let us compare for simplicity 10-methyl-1 : 2-benzanthracene with the analogous benzacridine (in which we shall now retain the benzanthracene numbering).




The modification of the charge densities $q_3$ and $q_4$ from the value unity in the parent hydrocarbon is obtained in both cases by using the values of $\pi_{3,10}$ and $\pi_{4,10}$ of Table I in equation (10) where $k$ in equation (13) is given a positive value for nitrogen substitution and a negative for $CH_3$. The fact that $\pi_{3,10}$ is considerably greater in magnitude than $\pi_{4,10}$ is essential to our argument. The methyl group produces a flow of charge to make $q_3 > 1$ and $q_4 < 1$. Bonding with the first electrophilic oxygen atom would therefore take place at position 3. The charge distribution of the two electrons forming the resulting C–O $\sigma$-bond in the plane of the molecule will presumably be asymmetric, with a greater density around the more electro-negative oxygen atom. However, this atom also possesses two electrons occupying an orbital of the same symmetry as the $\pi$ electrons of the main hydrocarbon skeleton. Conjugation will therefore take

place with the ring system giving the C–O bond some additional $\pi$-bond character. This results in a net flow of $\pi$-electron charge away from the originally doubly occupied oxygen orbital. It can be shown that much of this charge flows into the adjacent (ortho) positions. Thus atom 4 acquires charge to offset its initial deficiency, and hence becomes a reactive centre for the second oxygen atom. Now consider the benzacridine. The nitrogen atom produces a flow of charge to make $q_3 < 1$ and $q_4 > 1$. The significant point is that owing to the unequal values of $\pi_{3,10}$ and $\pi_{4,10}$ and under the assumption that $|k_N| \geqslant |k_{CH_3}|$ as is generally supposed, atom 4 is now driven negative less than was atom 3 in the methyl case, whereas atom 3 is now more positive than was atom 4 for methyl substitution. Both these factors operate less favourably for the Os $O_4$ reaction than in the case of the methyl derivative. The first bond is formed less readily at the less negative atom 4. The oxygen atom again donates electrons to the ring but atom 3, which was initially driven strongly positive, may remain so, or at any rate will become less negative than atom 4 in the corresponding methyl case. In consequence it may fail to act favourably as an attracting centre for the second electrophilic oxygen atom.

It is not seriously suggested that the reaction follows so well-defined a path, but in broad outline the argument seems to be quite reasonable. The above argument is not of course confined to the two molecules under discussion but can be applied quite generally to all derivatives.

*Application to the carcinogenic problem.*

The present calculations were undertaken in the first place with a view to examining the hypothesis put forward by Pullman (1947) that the carcinogenic activity of the polycyclic hydrocarbons is due to the high $\pi$ electron density of the so-called *K* region. In virtue of the rough approximations made in Pullman's (1947) treatment, especially in the quantities used to describe the electron density distribution, it seemed essential to carry out an independent examination of the hypothesis.

TABLE V.

| Molecule. | Method of application. | |
|---|---|---|
| | Skin. | Subcutaneous tissue. |
| 1 : 2-benzanthracene . . . . | 0 | 0 |
| 1′-methyl-1 : 2-benzanthracene . . | 0 | 0 |
| 2′- ,, ,, . . | 0 | . . |
| 3′- ,, ,, . . | 0 | . . |
| 4′- ,, ,, . . | 0 | . . |
| 3- ,, ,, . . | + | ++ |
| 4- ,, ,, . . | + | ++ |
| 5- ,, ,, . . | ++ | ++ |
| 6- ,, ,, . . | + | . . |
| 7- ,, ,, . . | + | + |
| 8- ,, ,, . . | + | 0 |
| 9- ,, ,, . . | ++ | +++ |
| 10- ,, ,, . . | +++ | ++++ |

Activity roughly proportional to the number of +.

We shall discuss this with reference to the results obtained for 1 : 2-benzanthracene and its derivatives whose $K$ regions correspond to the 3–4 bond and its associated atoms.

Table V, taken from a review by Badger (1948) on the carcinogenic activity of polycyclic hydrocarbons, lists the relative carcinogenic powers of monomethyl derivatives. Since the substituent group is the same throughout the series, it is possible to compare the electron configuration of the $K$ region of each substituent by direct reference to Tables I, II and III, since it is unnecessary to specify explicitly the energy parameter $k$ of equation (13) for the $CH_3$ group, remembering only that it is negative. The results indicate a decrease in 3–4 bond order upon substitution at all positions except $u = 3'$, so that the bond order decreases whilst carcinogenic activity increases. Moreover the diverse results obtained for the sum of the free valence polarisability coefficients permit no correlation with this quantity. There remains, as in the case of the osmium tetroxide reaction, a possible correlation with the total charge density, and here, provided we exclude substitution in the angular benzene ring, and at the $K$ region itself there exists a rough relationship between the total flow of charge into the $K$ region given by $+k\beta(\pi_{3,u} + \pi_{4,u})$ and the activity, though substitution at 9 gives a somewhat anomalous result (Table I).

Substitution in the angular ring provides, however, a series of results in direct contradiction to the proposed correlation. These results are as consistent and almost as numerous as those favouring the correlation. The metabolism of benzanthracene compounds gives products substituted in position 4′, and Boyland (1950) has proposed intermediate compounds involving positions 3′ and 4′ of the angular ring. It may be that substitution by $CH_3$ in these positions interferes with the metabolic process, and in some way destroys the potential activity of such derivatives. This would, perhaps, explain in part some of the inactive derivatives. But, in fact, there are other important exceptions which cannot be assumed, on this basis, to impair the metabolic process. For example, the 1′ : 10–, 2′ : 6–, 2′ : 7-dimethyl derivatives are non-carcinogenic, though the present results indicate an increase in $K$ region charge density over the corresponding carcinogenic 10-, 6-, and 7-monomethyl derivatives. The 1′ : 10-dimethyl derivative offers a particularly striking case when comparison is made between the magnitudes of the polarisability coefficients of the $K$ region for the 1′ and 10-positions (Table I). Clearly the small polarisability for the 1′-position should scarcely affect the large activation of the $K$ region due to the 10-substitution, and yet the highly active 10-methyl is converted to an inactive 1′ : 10-dimethyl derivative.

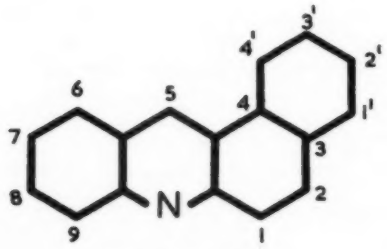
The results are also directly applicable to the benzacridine systems. We have already emphasised that the change of both bond order and free valence in passing from an alternant hydrocarbon to a derivative is independent of the electronegativity of the substituent group. In contrast the change in charge density depends directly upon the electronegativity, so that for all positions of substitution except $u = 8$ and 3, 4, the positions of the $K$ region itself, charge will flow into or away from the $K$ region according as the substituent is less (methyl) or more (nitrogen) electro-negative than carbon. Experimentally we find that in general methyl substitution produces or enhances activity, whilst aza substitution inhibits it. This is therefore further strong evidence that in all probability neither the bond order nor the free valence play an important

role in the activity of the *K* region. The results given in Table VI are taken from Badger's (1948) review. The numbering system peculiar to each benzacridine is used. It is interesting to note that the derivatives of 3 : 4-benzacridine show much less activity in general than the comparable derivatives of 1 : 2-benzacridine. This is in keeping with the results for 1 : 2-benzanthracene (Table I), which indicate a much greater reduction of the *K* region charge density for N substitution at position 10 than at position 9 (benzanthracene numbering).

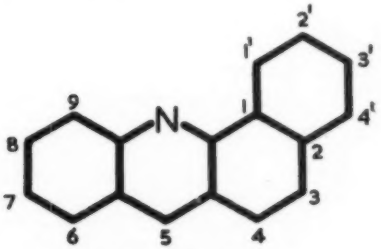
TABLE VI.

| Compound. | $\Delta Q$*. | Carcinogenic activity. Skin. | Subcutaneous tissue. |
|---|---|---|---|
| 1 : 2-benzanthracene | 0 | Active (Steiner and Falk, 1951) | |
| 3 : 4-benzacridine | −0·0387 | 0 | .. |
| 5-methyl-3 : 4-benzacridine | −0·0360 | 0 | .. |
| 7-methyl- ,, | −0·0393 | 0 | .. |
| 5 : 7-dimethyl- ,, | −0·0366 | 0 | .. |
| 5 : 8-dimethyl- ,, | −0·0233 | + | 0 |
| 5 : 9-dimethyl- ,, | −0·0329 | 0 | + |
| 5 : 7 : 9-trimethyl- ,, | −0·0335 | +++ | 0 |
| 1 : 2-benzacridine | −0·0027 | 0 | .. |
| 5-methyl-1 : 2-benzacridine | +0·0360 | +++ | . |
| 7-methyl- ,, | +0·0004 | 0 | .. |
| 5 : 7-dimethyl- ,, | +0·0391 | ++++ | +++ |
| 5 : 8-dimethyl- ,, | +0·0487 | ++++ | +++ |
| 5 : 9-dimethyl- ,, | +0·0354 | +++ | +++ |
| 5 : 7 : 9-trimethyl- ,, | +0·0385 | +++ | ++ |

* Calculated from Table I using $k_N = -k_{CH_3} = 1$.




3:4-BENZACRIDINE




1:2-BENZACRIDINE

Of the substituent groups which conjugate more freely than $CH_3$ and would produce a net flow of charge to the *K* region, the 10-hydroxy- and amino-derivatives are reported as inactive and active respectively. In contrast the 10-CN derivative, which must produce a flow of charge away from the *K* region, is a potent carcinogen.

The present results indicate therefore that a correlation between variation of carcinogenic power and variation of charge density in the *K* region does not hold in all cases. If we are prepared to admit that for some unknown reason substitution in the angular ring of 1:2-benzanthracene may inhibit potential activity of the *K* region, then for substitution in all other positions a measure of correlation holds, though not without some apparent anomalies.

To some extent these results confirm those obtained by Pullman (1947) and Daudel and Daudel (1950), using simpler but less accurate techniques, though the anomalies discussed above seem to have been ignored. However, these authors have suggested correlations of carcinogenic activity with various calculated quantities which within their own method of description bear some resemblance to the well-defined theoretical quantities used in the M.O. method. Of these correlations the only one which remains at all acceptable according to the M.O. calculations is that between the total charge density of the *K* region and the activity. We have shown that correlations with the bond order and total free valence of the *K* region when applied to derivatives no longer hold. It seems probable that adequate calculations based on the valence bond method would give the same sort of result.

If we suppose, therefore, that though the evidence given above is not decisive, the *K* region may play some role in the carcinogenic activity of these molecules, then the most important conclusion to be deduced from the present results is that the *K* region may act by a largely heterolytic mechanism involving attack by two electrophilic centres. In the absence of any detailed knowledge of the pharmacological action of these molecules a suggestion of this kind probably exceeds more than we can legitimately conclude. On the other hand, it is not an altogether unwarrantable deduction from our results, and is presented, not as a serious proposition, but rather that it may be of value in studies of these problems.

I should like to express my thanks to Professor C. A. Coulson for suggesting this problem and for his interest throughout its progress. Thanks are also due to Dr. J. van der Waals for checking some of the calculations, and to Dr. B. Pullman for providing alternative calculations of the $\pi_{r,u}$ coefficients. I am further indebted to the Department of Scientific and Industrial Research for a grant. A calculating machine was provided through the generosity of the British Empire Cancer Campaign.

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